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(54) Title: REGULATION OF OVARIAN MATURATION AND FUNCTION USING EPIDERMAL GROWTH FACTOR

(57) Abstract

A method is provided for regulating vertebrate ovarian maturation and function using growth factors by providing an amount of epidermal growth factor to prepubertal ovaries of female vertebrate species including stimulation of primordial follicles and enhancing activation of dormant follicles with the results that the mechanics of the method regulate early development of ovarian follicles. The method provides activation of dormant follicles and early ovarian maturation which is accelerated to the point of producing earlier breeding cycles as well as increasing first litter sizes. The method for regulating vertebrate/mammal ovulation maturation is also directed to increasing the ovulation rate and increasing litter size at an age of normal breeding.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/08192

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRUPEN C G ET AL: "Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development in vitro." REPRODUCTION, FERTILITY, AND DEVELOPMENT, (1997) 9 (6) 571-5. , XP000857263 abstract --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 November 1999	13/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US WEI Z ET AL: "Regulation of ovarian follicular development by epidermal growth factor in IVF superovulation cycles." retrieved from STN Database accession no. 1998259165 XP002124204 abstract & CHUNG-HUA FU CHAN KO TSA CHIH 'CHINESE JOURNAL OF OBSTETRICS AND GYNECOLOGY!, (1997 FEB) 32 (2) 87-9. , ---	17
P,X	CORTVRINDT R G ET AL: "Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotropin." FERTILITY AND STERILITY, (1998 DEC) 70 (6) 1114-25. , XP000857260 the whole document -----	1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/08192

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-17 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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YU	Yugoslavia
ZW	Zimbabwe

REGULATION OF OVARIAN MATURATION AND FUNCTION USING EPIDERMAL GROWTH FACTOR

Field of the Invention

The present invention relates generally to methods of regulating ovarian follicular development, maturation and ovulation to facilitate vertebrate mammalian reproduction. More specifically, the present invention relates to a method whereby Epidermal Growth Factor ("EGF") is administered to a vertebrate mammal to promote ovarian follicular development, maturation and ovulation and/or accelerate follicular differentiation in the preantral stages and increase the number of ova available for fertilization at each reproductive cycle. In another aspect, the invention relates to the treatment of prepubertal mammals and vertebrate subjects with EGF to regulate and accelerate ovarian maturation and function.

Background of the Invention

Many diagnostic and therapeutic procedures exist to aid reproduction practitioners in reproductive diagnostics, therapeutics and interventions. In mammalian embryology, mammalian oocytes enter the first meiotic division during fetal life, but become arrested in late prophase (in the dictate or diffuse diploid stage of meiosis) before or just after birth (Beaumont, H.M., et al., *Proc. R. Soc. London* (Series Biological Sciences) 155:557-579 (1962). Resumption of meiosis normally does not occur until shortly before the first ovulation, when previously unidentified growth factor trigger ovarian development, followed by a surge of gonadotropins prompts the resumption of meiotic maturation (Dekel, N., et al., *Proc. Nat'l Acad. Sci. U.S.A.*, 75:4369-4373 (1978).

Currently, infertility in humans ranges from approximately 10-15% of couples and the risk of infertility is doubled for women between the ages of 35-44 when compared to women between the ages of 30-34. In the United States, the majority of infertility can be accounted for by reproductive problems in the female.

In U.S. Patent No. 5,395,825, investigators provided methods for determining swine ovarian follicular developmental stages. Laminin is a basement membrane protein that must be synthesized and secreted as the follicle grows. Thus, the development of laminin may be used as a specific for granulosa cell differentiation and development in early stages of follicular growth. In another study, published by Vinter-Jensen et al., in 1995, EGF

administered to mini-pigs was found to stimulate growth of heart, liver, and urinary tract tissue. Reproductive organs, however, were not closely evaluated in any of these studies. Eppig and O'Brien published data in 1996 which showed that EGF treatment of neonatal mouse ovaries, cultured *in vitro*, yielded an increased the number of eggs recovered for *in vitro* maturation and fertilization. This data also implicates a role for EGF in follicular development. Breider, *et al.*, studied the effect of intravenous infusion of EGF into mature rats and found that EGF stimulated growth and proliferation of may different types of tissues, including the mature rat ovaries, which exhibited increased ovarian weight, accompanied by the increase in the number of corpora lutea found in these ovaries. The corpora lutea is a structure that results from the ovulation of a mature antral follicle. Thus, in the mature rat, there is evidence that EGF administration increases the number of mature ovulations. Again, these studies were done in live animals suggesting the feasibility of using EGF *in vivo*.

Epidermal Growth Factor is a peptide hormone that stimulates the growth and differentiating of epidermal tissues during embryogenesis (Carpenter, G., et al., *Exper. Cell. Res.* 164-1-10 (1986); Kris R. M. et al., *Bio-Technol.* 3:135-140 (1985)). EGF may be purified from natural sources or may be obtained through application of recombinant DNA technology. EGF is a 53-residue polypeptide (M, -6000) that is mitogenic for a variety of cell types both in vivo and in vitro (Carpenter & Cohen, 1979). EGF was originally purified from the male mouse submaxillary gland (SMG) (Cohen 1962) and subsequently from human urine (Cohen & Carpenter, 1975; Gregory, 1975). Antibodies raised against mouse or human EGF are used to confirm expression in tissues or body fluids using immunoassays or immunocytochemical staining. Highest levels of EGF have been found in SMG (mouse), kidney, pancreas, duodenum, urine and milk (see Carpenter, 1985; Gregory, 1985; Burgess, 1989; Fisher & Lakshmanan, 1990). However, there is little information regarding EGF expression in other species because antisera against EGF show very little cross-species reactivity (Gregory, Holmes & Willshire, 1979; Schaudies & Savage. 1986) necessitating the development of homologous immunoassays (Joh. Itoh, Yasue et al. 1989). Despite their limited immunological cross-reactivity, both mouse and human EGF bind to cellular receptors on various cell types from several species with very similar affinities and efficacy (see Carpenter & Cohen, 1979; Carpenter, 1987). While heterologous radioreceptor assays are therefore possible, they lack specificity since other polypeptides (*e.g.* transforming growth factor alpha ("TGFα") are known to bind to the same receptors (see Burgess, 1989;

Massague, 1990). In addition, indirect modulation of EGF receptor affinity by heterologous ligands has been widely reported (see Schlessinger. 1986).

A homologous radioimmunoassay for the measurement of EGF levels in pig tissues and body fluids has been developed using an antiserum to recombinant porcine EGF. The assay is highly specific, showing no cross-reactivity with a variety of other polypeptides including the structurally related protein, transforming growth factor-alpha ("TGFα"). Furthermore, less than one- percent cross-reactivity was observed with mouse EGF emphasizing the necessity for homologous assays for EGF measurement. immunoreactive EGF was present in extracts of pig kidney and pancreas (3.44 +1- 0.43 and 0.76 $\pm$ 0.13 (S.E.M.) pmol/g wet weight respectively), but was not detected in extracts of submaxillary gland or liver. Although immunoreactive EGF was not detectable in uterine, allantoic or ovarian follicular fluids, colostrum contained EGF at biologically active concentrations. Immunoreactive EGF was also present in pig urine, with similar concentrations in samples from male or female animals. In addition, pig urine inhibited the binding of iodine-labeled EGF to 3T3 fibroblasts and stimulated DNA synthesis in quiescent monolayers of these cells, indicating that the immunoreactive material in urine is biologically active. Quantitative comparisons of the data presented here with that published previously indicate considerable species variation in the EGF levels of various tissues and body fluids.

In follicular development, the development of a dormant primordial follicle into a large mature follicle must occur before the ultimate mature follicle is stimulated to ovulate and produce ova capable of fertilization. This general process is the key for the production of eggs in many species. Thus, the ovary functions as a reservoir of dormant follicles and through the process of follicular development, some of these dormant follicles will develop and mature to produce hundreds to thousands of eggs. Follicular development can be envisioned as a pipeline and the control of the process occurs by many regulatory steps. In the later stages of follicular development, it is known that Follicle Stimulating Hormone ("FSH") is important for growth and development of mature antral follicles and ultimately Lutenizing Hormone ("LH") stimulates the ovulation of a mature follicle and the production of the egg. However, the factors regulating early steps of follicular development have been unknown.

The present invention provides methods to influence the early regulatory steps that control ovarian follicular development in vertebrate and mammalian species. The present

invention, may, for example, be utilized in bovine, equine, porcine, canine, feline, human mammals, avian, aquatic, reptilian species or the like. It is contemplated that the present invention will directly benefit the production of food species, such as bovine, swine, and avian and aquatic food species, such as chicken, turkey, duck, salmon, cod, trout or the like, in other farm animals, assist in human infertility, and in reproduction of endangered species and zoo animals.

Summary of the Invention

The present invention is directed to a method of initiating and regulating ovarian follicular development in mammalian females. In the present invention, rabbits and pigs were used as vertebrate mammalian models. Genetic markers have been identified which are initially transcribed in activated primordial follicles in rabbits and pigs. Expression of two rabbit zona pellucida genes R55 and R75, were localized in prepubertal rabbit ovaries by in situ hybridization. Results indicate that transcription of the genes occur initially in activated follicles and increases through early stages of follicular development. Expression of the R55 and/or R75 genes offers a qualitative method to identify activated follicles *in vivo* and a quantitative method to activate follicular development *in vitro*.

In addition to permitting identification of morphological changes and granulosa cell proliferation, these gene markers provide functional definitions for the initial steps in folliculogenesis. To determine whether growth factors can stimulate activation of primordial follicles, tissue explants from immature rabbit ovaries (14 days old) were cultured with or without mouse EGF. Ovaries at this age contain only primordial follicles providing an ideal population in which to study activation of follicular development. The relative amounts of R55 mRNA were measured by Northern blot analysis. The Northern blot assay results indicated that EGF (50 ng/ml) increased expression of R55 in primordial rabbit ovarian follicles. It was concluded that EGF stimulates expression of zona pellucida genes in primordial follicles and enhances the level of activation of dormant follicles.

The ability to regulate both the timing and the magnitude of follicle activation can influence the overall reproductive capacity of a given female and lead to new methods for managing reproductive function in clinical or agricultural settings.

In another aspect, the present invention is directed toward using EGF in swine to regulate ovarian follicular development, ovarian maturation and ovulation. Swine production is limited, in part, because during early breeding cycles relatively few ova are ovulated,

resulting in small litter sizes in young sows. Thousands of ovarian follicles containing eggs are formed in new born animals and are available to be activated, but for unknown reasons only a few mature in early estrus cycles. By accelerating earlier activation of dormant ovarian follicles and earlier ova maturation, the present invention provides a method for enhancing productive efficiency in sows accelerating breeding cycles and increasing earlier litter sizes.

Rabbits have ovarian development patterns similar to pigs. The rabbit model was employed to model swine ovarian development. Rabbit test results indicate that activation of dormant follicles and expression of the rabbit R55 zona pellucida gene are increased approximately three fold by treatment of prepubertal ovaries with EGF. Pig follicles synthesize a zona pellucida molecule, ZP3α, which is 74% identical to rabbit R55 and is detectable with molecular probes to R55. This molecular marker for activation and techniques established in the rabbit model was used for studies with pig ovaries.

Confirming these findings in two distinct mammalian species, namely rabbit and swine, suggests a commonality to all vertebrates and mammals that the mechanism of early follicular activation may be accelerated by administering EGF.

Brief Description of the Figures

Fig. 1 is a diagram showing a generalized overview of follicular development;

Fig. 2 is a schematic diagram presenting the many potential steps and control points for follicular development;

Fig. 3 is a diagram showing the later stages of follicular development which occur at growing follicles when they respond to stimulation by FSH and where they mature into antral follicles;

Fig. 4 is a diagram that shows the different stages of follicular development that are present in prepubertal rabbits;

Fig. 5 is a graph demonstrating the first wave of follicular development in prepubertal rabbits;

Fig. 6 is a photograph representation showing the Northern blot analysis of R55 in the immature rabbit ovaries;

Fig. 7 is a graph showing R55 expression in developing rabbit ovaries;

Fig. 8 is a composite representation showing the localization of R55 protein and messenger RNA in developing ovaries of prepubertal rabbits;

Fig. 9 is a diagram showing that a second ZP gene in the rabbit R75 is a valid marker for activation of primordial follicles and its pattern of expression which is similar to R55;

Fig. 10 is a graph showing the effects of growth factors (epidermal growth factor, EGF) on R55 expression in immature rabbit ovaries;

Fig. 11 is a representation to ovarian development in prepubertal pigs;

Fig. 12 presents the follicle populations present in the immature pig ovaries at day 70 postpartum or 10 weeks of age.

Fig. 13 is a schematic depicting the follicle development that occurs during ovarian maturation in the prepubertal pig;

Fig. 14 presents a schematic that shows the reproductive maturation timeline as relative to the situation found with commercial gilts;

Fig. 15 is a graph showing the inventive results established in the rabbit was applicable to the pig;

Fig. 16 is a graph showing the effect of EGF on ZP expression in isolated primordial porcine or pig follicles.

Fig. 17 is a diagram summarizing overall basic working model for acceleration of follicular development in accordance with the invention.

Fig. 18 is the DNA and amino acid sequences of the recombinant porcine EGF (rec-pEGF) protein, which is expressed, in the bacterial system.

Fig. 19 is a representative sample of 15% polyacrylamide SDS gels of expressed rec-pEGF protein purified in Ni-Agarose columns.

Fig. 20 are Western blots of rec-pEGF, in which panel A is a coommassie stained 1D-PAGE gel and panel B is mouse EGF antibodies detected with chemiluminescence.

Fig. 21 is a graph of a fibroblast proliferation assay using tritiated thymidine uptake to determine the effect of increasing rec-pEGF concentrations on DNA synthesis.

Fig. 22 is a graph depicting the average size of the ten largest follicles observed in H/E-stained sections of pig ovaries, comparing the EGF administered group, with a control group and a group administered neither the EGF or a placebo.

Fig. 23 is a graph illustrating the number of growing ovarian follicles at 70 days of age for the EGF administered group, a control group and a group administered nothing.

Detailed Description of the Invention

Swine sows are polyestrous with a period of lactational anestrus occurring until after weaning. The estrus cycle length averages 21 days. Return to estrus occurs after the recovery period, or 4-7 days post-weaning. Estrus onset is marked by the preovulatory surge of LH. Gilts come in to first estrus around eight months of age. Estrus (heat behavior) occurs for 2-3 days, averaging 60 hours in mature sows, but is only about 48 hours long in gilts. Ovulation actually occurs during the last third of estrus.

Ovulation occurs 36 to 42 hours after the onset of standing heat in mature sows, about 12 hours earlier in gilts. On average, 10-20 ova are shed from follicles ranging from 0.7 to 1.0 cm. Fecundity is highest in sows at 2 to 4 years of age. Gilts average 10-15 ova per cycle. Embryonic survival rate is 65%-75%, resulting in litter sizes of 8 to 12 piglets. Heritability of litter size is low, but tremendous breed differences exist. Recent studies suggest fecundity may be linked to the presence of a specific estrogen receptor. In addition, fecundity is strongly linked to lactational feed intake. Limiting feed intake in gestation leads to greater feed intake during lactation. High feed intake in lactation results in good milk production, high piglet weights at weaning, short wean to estrus intervals, and maximal ovulation rates.

The zona pellucida (ZP) matrix surrounding oocytes appears in follicles following activation, indicating transcription of ZP genes is specifically development. The protein and mRNAs for the rabbit 55 kD ZP component, R55, are initially expressed in oocytes of activated follicles before morphological changes. Preliminary results indicated that pig ZP proteins are expressed in activated follicles and show that expression of the pig homologue to R55, ZP3α, provides a marker for determining initiation of follicular development in this species. An objective of the present invention is to determine that EGF stimulates activation of pig primordial follicles, subsequently resulting in expression of ZP genes by oocytes and accelerates the early stages of follicular development. Thus, ZP markers can provide an objective method for measuring follicle activation and development in addition to more subjective observations based on morphological changes. Furthermore, these experiments are necessary to determine effectiveness for using EGF to accelerate prepubertal ovarian development in neonatal pigs and ultimately increase reproductive efficiency. An increase of even 10% of pigs per litter would be economically significant for commercial pork producers.

Before follicular development begins, ovarian follicles are termed "primordial" or "dormant" follicles and consist of undifferentiated squamous granulosa cells surrounding an oocyte arrested in prophase I of meiosis. When activated, the granulosa cells proliferate and

differentiate, while the oocytes enter a growth phase. During these early steps of follicular development ZP proteins (rabbit R55 and pig ZP3α) are synthesized and assembled around the oocyte. Recent studies have begun to use culture systems to study regulation of these early stages of folliculogenesis. In preliminary observations R55 sense (+) and antisense (-) RNA probes were labeled with [35^s]-UTP and used for *in situ* hybridization to determine the spatio-temporal pattern of expression of R55 during early rabbit ovarian development. Ovaries were collected from prepubertal rabbits (14 and 28 days postpartum, d.pp.), fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for localization of R55.

Localization of R55 protein and mRNA in activated and growing follicles of prepubertal rabbit ovaries was demonstrated. In 14 d.pp. ovaries, R55 protein and mRNA were localized in oocytes of some primordial follicles but are undetectable in the majority of primordial follicles. It was illustrated that R55 protein and mRNA are abundant in oocytes of primary follicles from 28 d.pp. animals. Expression of R55 was incre*ased* in transitional follicles that exhibited characteristics of both primordial and primary follicles. In the cortex of ovaries from 28 d.pp. animals, many of the primordial follicles closest to the medullar region of the ovary are expressing R55 while it is undetectable in the cortical follicles. The position of these labeled primordial follicles indicated that they are the activated group of follicles that will develop during the peripubertal period of folliculogenesis. This spatio-temporal pattern of expression for R55 indicates that transcription of this gene is initiated in oocytes during the activation of follicular development.

Rabbit Ovary Culture

A method to culture pieces of ovaries from 14 d.pp. rabbits was developed, modeled after techniques used for ovaries from neonatal mice and bovine embryos. Tissue pieces (1-2 mm^3) from 14 d.pp. rabbit ovaries containing only primordial follicles were placed in cell culture inserts with 3.0 mm pores and cultured in 24-well plates with 300 microliters medium (50-50 blend of (a) F-12 Nutrient Mixture (Ham)(1X), liquid contains L-glutamine, and (b) Medium 199 (lX), liquid contains Earle's modified salts, 1.250 mg/L sodium bicarbonate, and L-glutamine.

Rabbit Ovary Cultures: Treatment With EGF

Cultures were treated with mouse EGF (50 ng/ml) and analyzed for the level of expression of R55 mRNA. Treatment with EGF produced a 3-fold increase in amount of R55 mRNA (normalized to 28S) as compared to controls incubated in medium alone (Figure 3). These results suggest that EGF stimulates activation of follicular development and expression of R55 in dormant primordial follicles.

Swine Ovary Cultures: Treatment With EGF

For *in vitro* experiments, ovaries from prepubertal pigs (3-5 weeks post partum) were collected for tissue cultures of ovarian pieces. The ovaries contained a large population of dormant primordial follicles similar to 14 d.pp. rabbits. Pig ovarian tissue cultures were treated with EGF at different doses for 6 days and tissues collected for *in situ* hybridization and Northern blot analysis of ZP3α. The 6 day culture period was chosen because in preliminary experiments with cultures of rabbit ovarian tissue, treatment with EGF (50 ng/ml) produced significant increases in expression of R55.

In Vivo EGF: Swine

For *in vivo* experiments, prepubertal pigs at different ages (1, 35, and 70 d.pp.) were treated with subcutaneous osmotic pumps delivering constant daily dosages of EGF for fourteen days. Ovaries were removed following treatments to evaluate follicle development. In ovarian tissue from *in vitro* and *in vivo* experiments, expression of ZP3α mRNA was localized by *in situ* hybridization and the percentage of activated primordial follicles determined. The amount of ZP3α mRNA in cultured pig ovarian tissue was determined by Northern blot analysis to quantitate the level of ZP expression (normalized to levels of 28S RNA).

Two groups of young female pigs were administered recombinant porcine EGF (rec-pEGF). Group 1, aged 30 d.pp., were given 540 μg/day rec-pEGF using subcutaneous osmotic pumps, for fourteen days. A control group of the same age were administered the injection vehicle only. A non-treated group was maintained under identical conditions as the control group and Group 1. Group 2, aged 21 d.pp., were given 600 μg/day rec-pEGF for fourteen days.

After fourteen days post-treatment, the Group 1 pigs were necropsied and histological evaluation of ovarian sections (hematoxylin/eosin stained) revealed larger ovarian follicles and the presence of a 70% greater population of growing follicles in the rec-pEGF treated animals than in the control or non-treated groups.

The Group 2 pigs were grown to 10 weeks of age, then necropsied. At the time of necropsy, the pigs receiving rec-pEGF showed no negative effect on weight gain or organ development. The ovaries of the Group 2 pigs showed an almost 100% increase in size when the tissues were collected at 10 weeks of age, when compared to the control group and the group treated with nothing. Figures 22 and 23 set forth the foregoing results of the Group 1 and Group 2 pigs.

All pigs in Group 1 and in Group 2 were dosed using subcutaneously placed osmotic pumps. During the time of rec-pEGF, both groups were individually housed, with general health, feeding and behavioral observations made regularly and recorded.

Summary of In Vitro and In Vivo Results

The foregoing tests demonstrate that EGF stimulates expression of ZP3α and activates primordial follicles *in vitro* and in whole ovarian cultures. EGF stimulates activation of dormant pig follicles. EGF (50 ng/ml) stimulates DNA synthesis in granulosa cells from primary pig follicles and expression of FSH receptors in granulosa cells from antral pig follicles.

In pigs, an activated primordial follicle matures to antral stage in approximately 84 days and from activation to ovulation will be about 100 days. Therefore, normally follicles that ovulate in the first cycle were activated around day 50 post partum. The process can be accelerated (as we have shown) by application of EGF before day 50 post partum to activate ovarian and follicular development. The inventive goal is to increase the number of primordial follicles activated earlier in prepubertal development, thus increasing the number of growing follicles. Subsequent treatment with gonadotropins will result in more mature follicles at earlier ages in young sows. Further development of this model should result in larger and earlier litters in production sows.

Experiments on pigs demonstrate that pig ZP3α is expressed during activation of dormant primordial follicles and determines the period of prepubertal ovarian development during which follicle activation is greatest. In prepubertal pig ovaries, dormant primordial

follicles are located in the outer portion of the cortex while activated and growing follicles are found close to the medullar region. Since ZP3α is expressed in activated follicles, its mRNA is localized in primordial follicles closest to the medullar region and labeling is more intense in the transitional and primary follicles. Thus the labeling pattern will appears as a gradient of signal, undetectable in the cortex and more intense toward the medullar region.

Figure 1 is the diagram showing a generalized overview of follicular development This diagram depicts the development of a dormant primordial follicle into a large mature follicle. Ultimately the mature follicle may be stimulated to ovulate and produce a fertilizable egg. This general process is the key for the production of eggs in many species.

Figure 2 is a schematic diagram representing the many potential steps and control points for follicular development. The ovary is depicted as a reservoir of dormant follicles and through the process of follicular development these dormant follicles will develop and mature to subsequently produce hundreds to thousands of eggs. Depicted is follicular development as a pipeline and the control of this process consisting of many valves or potential regulatory steps. In the later stages of follicular development, it is known that FSH is important for growth and development of mature antral follicles. Ultimately, LH stimulates the ovulations of a mature follicle and the production of the egg. However, the factors regulating early steps of follicular development have been essentially unknown for many years. The present invention was developed as an understanding how some of these early regulatory points are controlled in ovarian follicular development.

Figure 3 is a diagram showing the later stages of follicular development that occur in growing follicles when they respond to stimulation by FSH where they mature into antral follicles. These are readily observable by morphological changes in histological sections of ovaries. However, in the early stages of follicular development the morphological changes are not as obvious during the activation of dormant primordial follicles. Before questions about the regulation of the activation and development of these early stage follicles could be focused. Identity of genetic markers which could be detected and measure molecular changes in activated primordial follicles was necessary. These markers are genes that are turned on in the process of activation of dormant follicles that can be observed before any morphological changes take place. One family of genes that was known to be expressed in early stages of follicular development is the one for the zona pellucida. The zona pellucida is the glycoprotein matrix (ring around egg) that surrounds the mature oocyte. Note the lack of

protein matrices which surrounds the egg in the dormant follicle and the outer line in the growing follicle that indicates the presence of zona pellucida.

Table 1 lists all the genes for zona pellucida proteins that are present in four species. the rabbit, pig, mouse, and human. These have all been cloned and published. The zona pellucida gene that was chosen to investigate was the R55 gene in rabbit. The homologue this gene in the pig is ZP3α and there are similar genes present in the mouse and a human known as ZP1. First studies were designed and carried out in the rabbit so those studies are described first; R55was studied to determine if this gene fits criteria as a early to marker or indicator for activation of dormant primordial follicles.

TABLE 1

ZP Nomenclature

Rabbit:	R45, R55, and R75
Pig:	ZP3β, ZP3α, ZP2 and ZP1
Mouse:	ZP3, ZP2, and ZP1
Human:	ZP3, ZP2 and ZP1

Figure 4 shows the different stages of follicular development that are present in prepubertal rabbits. This is one of the reasons this species was selected for study. In prepubertal rabbits, ovarian development occurs after birth (humans are more like pigs in their temporal developmental profile). Because of this fact, ovaries from different ages of prepubertal rabbits were taken and with more mature follicles at each age. As shown in the fourteen day post-partum rabbit (which means fourteen days after birth) the ovary is essentially a bag of primordial follicles. At 28 days post-partum, a group of follicles have begun to develop and resulting in a population of primary follicles, which are morphologically different from the primordial follicles in that they contain a single layer of cuboidal granulosa cells surrounding the follicle. 42 days post-partum secondary follicles are present indicating yet another step of maturation in the early development of follicles. Moreover, by 56 days post-partum, tertiary follicles begin to appear which represent early stages of antral development in the ovarian follicles. The expression of the R55gene is linked to the activation in early development of ovarian follicles, *i.e.* its pattern of expression following this initial wave of follicular genesis.

Figure 5 graphically demonstrates the first wave of follicular development in prepubertal rabbits. A peak in the percentage of follicles initiating development (primary follicles) is seen around 6 weeks of age. These data are based on morphological characterization of histological sections and are presented in Table 2.

TABLE 2

Percentage of follicles present in rabbit ovaries of different ages.

	2wk	4wk	6wk	8wk
Primary	0	11	19	4
Secondary	0	0	6	11
Tertiary	0	0	0	3
Primordial	100	89	75	80

Figure 6 shows the northern blot analysis of R55 in immature rabbit ovaries. In this study. total RNA was isolated from different age rabbit ovaries, separated on agarose gels and transferred to biotrans nylon membranes. The membrane was then probed with a cDNA probe for R55 labeled with P^{35} and developed by autoradiography. As shown in panel A, R55 is undetectable at 14 days postpartum. A more intense band at 28 days postpartum and a very dark band at 42 days postpartum is presented. The R55 band is less intense at 56 days postpartum. This blot was then stripped and reprobed with a constitutive gene EF1α to show equal loading and transfer of the RNA. This experiment was repeated with 3 different groups of RNA. The intensity of the bands were determined by optical density and the data shown graphically.

Figure 7 is a graphic showing R55 expression in developing rabbit ovaries. The pattern of R55 expression correlates with the pattern from the first wave of folliculogenesis in the prepubertal rabbits as shown in Figure 5. This graph was obtained by determining the ratio of R55 to EF1α from each sample and then this number was graphed as a relative percentage of the maximum R55 expression found. Data supporting the graph of Figure 7 are presented in Table 3.

TABLE 3

R55 expression in rabbit ovaries from different ages.
Percent of maximum expression of R55 mRNA was determined on Northern blots.
Maximum expression occurs at 42 days postpartum (d.pp.)

d.pp.	14	28	42	56
% Max R55	3.1	36	100	45
SEM	1.8	9.8	0	7.3

Figure 8 is a composite showing the localization of R55 protein and messenger RNA in developing ovaries of prepubertal rabbits. Column 1 is the localization of R55 protein as determined by antibody staining with DAB conjugate. Columns 2 & 3 are in situ hybridizations for R55 messenger RNA. Column 4 is the in situ control using the sense strand as the riboprobe. The anti-sense strand was used in columns 2 and 3, all of which were labeled with S^{35}.

In the first row a single primordial follicle labeled for R55 protein and mRNA is shown. Based on hypothesis it is shown that this primordial follicle although it has not undergone any morphological changes has begun to develop since it is it expressing the R55 gene.

In the second row, it is shown that the primary follicles that appear by 28 days postpartum are expressing significant amounts of R55 protein and mRNA. Also important in this section shown by the arrow is a follicle, which we would term to be an intermediate follicle. It is somewhere in transition between the stages of primordial and primary and again as we would expect it is expressing a significant amount of R55. This is consistent with our prediction. In the third row is a section taken from a six week old rabbit (42 days postpartum). In this section, we show a secondary follicle that contains abundant amounts of zona pellucida protein beginning to form in a matrix around the oocyte in the first panel.

In the second and third panels, it is shown that the oocyte contains a large quantity of mRNA for R55. Not shown in this section are the many primary follicles that are present in 42 days postpartum that are also expressing abundant levels of R55. At 56 days postpartum in the fourth row, a developing follicle is shown and by this stage, a thick well developed matrix has formed around the oocyte that is a dark layer around the oocyte. In the second and third panels, it is shown that the amount of mRNA for R55 is dramatically decreased from what is seen in secondary follicles.

Returning to initial study steps of follicular development, a marker R55is initially expressed in activated follicles in the rabbit ovary. Figure 9 shows that it was determined that a second ZP gene in the rabbit R75 is a valid marker for activation for primordial follicles in its pattern of expression is similar to P55 so this gives two genes which can be measured in follicles to determine whether they are activated or not. Additionally, in rabbit two other genes were identified which are specific to granulosa cells and are expressed in these cells

during the transition from primordial to primary follicles. (Cx43 (connexin 43) and Inhibin-alpha). Now with these markers in hand questions about potential regulatory factors that might stimulate or inhibit activation dormant primordial follicles were considered.

Figure 10 shows the effects of growth factors EGF on R55 expression in immature rabbit ovaries. Ovaries were collected from 2 week old rabbits and the tissue mince into small pieces (-1mm^3). These ovarian explants were then cultured for 6 days in the presence or absence of EGF *(50* ng/ml). Total RNA was isolated and the relative amount of R55mRNA measured by Northern blot analysis. The result of 3 experiments were averaged and graphed. The level of P55 expression in the controls was defined as one. The level of expression of the epidermal growth factor treated samples was approximately 3 fold of that seen in the controls. This indicates that with treatment of epidermal growth factor there was a significant increase in the expression of R55 in these ovarian cultures. This effect is due to an increase in the number of follicles being activated. Data supporting the graph of Figure 10 are presented in Table 4.

TABLE 4

Results of 3 experiments showing stimulation of R55 mRNA expression by EGF. Values are the ratio of R55 to 28S optical densities from Northern blots.

	Control	EGF (50 ng/ml)
Exp. 1	1	2.87
Exp. 2	1	3.09
Exp. 3	1	3.28
Average	1	3.08
Std dev.	0	0.20518285
SEM	0	0.11846237

Figure 11 is directed to ovarian development in prepubertal pigs to determine if the inventive method would be applicable in other species. Figure 11 shows comparison of 10 week old pig ovaries to ovaries that are typical of a mature sow. In the rabbit, much (but not all) of the ovarian development occurs after birth. In the pig, ovarian development overlaps both the rabbit and human in developmental timetable. Development occurs over an extended time such that at 10 weeks the ovaries are very small and relatively immature. In pigs, antral follicles are normally not seen until 140 to 150 days (20 weeks) of age. This timetable may be accelerated by application of systemic EGF.

Figure 12 shows the follicle populations present in the immature pig ovaries at day 70 postpartum or ten weeks of age. In the two panels on the left, it is shown that a large population of primordial follicles are present in the cortex of ten week old pig ovaries. These represent the dormant or resting pool of primordial follicles, which will ultimately give rise to all the eggs that the gilt will produce throughout her life. In the two panels to the right, it is seen that in these same ovaries many secondary follicles are growing and developing. Based on morphology the 10 week pig ovary is very similar to the 42 day postpartum rabbit ovary.

Figure 13 diagrammatically depicts the follicular development that occurs during ovarian maturation in the pig. At birth, the ovary is primarily filled with dormant primordial follicles. Through the first 5 months of the gilts life the ovary matures and develops through the stimulation of endogenous growth factors and hormones such that at 5 months of age large population of antral follicles exists. These antral follicles are ready to be ovulated and will produce the eggs that are ovulated during the first heat.

Figure 14 shows the reproductive maturation timeline as relative to the situation found with commercial gilts. As depicted from birth through the first 5 months the ovary develops and matures. At about this time the gilt will undergo her first heat. At the first heat there is typically 10 to 12 eggs ovulated. After another 21 days, the gilt will enter her second heat at which time there will be slightly more eggs ovulated, on the order of 14 to 16 eggs. After another 21 days, the gilt will enter her third heat at which there will be 20 to 22 eggs ovulated and at this point, it is the recommended time for first breeding of a gilt. In this diagram, we can emphasize two obvious goals for the inventive method to accelerate ovarian maturation. One would be to increase the number of eggs ovulated in the first or second heat, such that commercial pork producers could then breed at these earlier times. Thus., saving time and feed costs while getting the most (larger litters) out of their immature gilts. Secondly, the inventive process could potentially shorten the period of time, the 5 month period, the gilt requires to reach the first heat. Both of these results combined would result in significant savings for the pork producer, with a concomitant increase in pigs per liter earlier in the reproductive cycle of the pig.

Figure 15 shows the inventive results established in the rabbit was applicable to the pig and it was determined to look at the effect of growth factors, specifically EGF, on ZP expression in immature pig ovaries. For this first experiment, ovaries were collected form pigs at three to five weeks of age at which the pig ovaries are very immature. The ovaries

were then minced into small pieces and grown in ovarian explant cultures with or without EGF (similar to what we have done with the rabbit ovaries). After 6 days of culture the tissue was collected and in this case proteins from these samples were isolated and solubilized. The amount of total zona pellucida protein in these samples was determined using an antibody we developed in guinea pigs against total zona pellucida proteins. The amount of porcine zona pellucida proteins in these samples were determined by dot blot analysis and quantified by optical density. The relative amount of zona pellucida protein is graphed for each sample (control and EGF treated 50 ng/ml). This was done for 3 replicates. These studies resulted in approximately a two fold increase in the amount of zona pellucida protein in the EGF treated ovarian explants. These results were consistent with the stimulation of P55 expression which shown in the rabbit ovarian explants. Data supporting the graph of Figure 15 is presented in Table 5.

TABLE 5

Stimulation of pig ZP proteins with EGF. Optical density of ZP proteins from ovarian explant cultures as measured by Protein dot blot analysis.		
Group ZP	OE EGF 50	OE control
	6355	2493
	6086	3763
	7193	3160
Avg. ZP	6544.66667	3 138.66667
Std. Dev.	577.358063	635.26871
SEM	333.337833	366.77256

Figure 16 graphically presents the results of studying the effect of EGF on ZP expression in isolated primordial follicles. As in the previous experiment, ovaries were collected from immature pigs at 3 to 5 weeks of age. In this case the ovaries were minced and the follicles enzymatically separated from the connective tissues and isolated. The population of small primordial follicles were collected and grown in cell culture well inserts. These were cultured for six days in medium alone as control or in medium supplemented with 50 nanograms per ml of EGF. After 6 days of culture the follicles were collected and proteins isolated and solubilized. The amount of total zona pellucida protein in these samples was determined using an antibody developed in guinea pigs against total zona pellucida proteins. The amount of porcine zona pellucida proteins in these samples were determined by dot blot

analysis and quantified by optical density. The relative amount of zona pellucida protein is graphed in Figure 16 for each sample (control and EGF treated 50 ng/ml). This was done for 3 replicates. As predicted from the hypothesis there was a significant increase in the expression of zona pellucida proteins in the primordial follicles treated with EGF. This is consistent with the hypothesis that EGF can stimulate activation of dormant primordial follicles as indicated by the expression of zona pellucida genes. Data supporting Figure 16 is presented in Table 6.

TABLE 6

Stimulation of pig ZP proteins in isolated primordial follicles with EGF. Optical density of ZP proteins from cultured primordial follicles as measured by Protein dot blot analysis.		
Group ZP	Primordial & EGF 50 ng/ml	Primordial, control
	1815	230
	371	9.85
	1697	0
Avg. ZP	1294.33333	79.95
Std. dev	801.8038	130.040407
SEM	462.92164	75.0788641

Figure 17 summarizes overall basic working model for acceleration of follicular development. Growth factors, specifically EGF, applied in early follicular development results in the acceleration of this process. In other words EGF stimulates activation of dormant follicles and accelerates follicular development at early growth stages of the animal. Increased numbers of activated follicles yield an increased number of follicles present and potentially available for maturation under the stimulation of FSH into large mature antral follicles, which at the LH surge would be ovulated. The result is the production of increased numbers of ovulated eggs and a shorter period of follicular development. This process can be applied to prepubertal development in the gilt (young sow) or other vertebrates to initiate or accelerate ovarian maturation and result in two positive effects. One being the increase in the number of eggs in early heats for the gilts and other species and two, possibly a shorter period of time to reach the first heat in these animals such that pork producers may be able to breed earlier and get more pigs per litter earlier in these animals.

Figure 18 is the DNA and amino acid sequences of encoding rec-pEGF protein. Rec-pEGF was expressed in the QiaExpress plasmid vector pQE30 . The DNA sequence

encoding pEGF begins at the adenine residue 36, with the AAT codon, encoding N^{13}, and ends at the cytosine residue 193 and the codon encoding Tyr^{65}. The DNA and amino acid sequence contains the 6X histadine tag at the amino-terminal end, and 19 amino acids from the bacterial plasmid vector at the COOH-terminal end. The 53 amino acids of the rec-pEGF is 100% identical to the published sequence for rec-pEGF published by Pascall, J.C., et al., *J. Mol. Endocrinol.* (1991) 6:63-70, which is hereby incorporated by reference as teaching a method for obtaining rec-pEGF using yeast and bacteriophage vectors.

Figure 19 is the results of gel electrophoresis on 15% polyacrylamide SDS gels, of expressed rec-pEGF protein purified on a Ni-Agarose column, showing a representative sample of some of the gels showing two purified samples of rec-pEGF. Lane 1 is a gel showing flow through of unbound bacterial proteins from a first sample run. Lane 2 is a gel showing the results after passing a wash buffer through the column. Lane 3 shows a relatively pure sample of rec-pEGF at a relative molecular weight of about 10 kDa, corresponding to the predicted molecular weight from the amino acid sequence of Figure 18. Lane 4 is a flow through sample of unbound bacterial proteins from a second sample run. Lane 5 is a gel showing the results after a wash buffer is passed through the column. Lane 6 shows a relatively pure sample of rec-pEGF at a relative molecular weight of about 10 kDa, corresponding to the predicted molecular weight from the amino acid sequence of Figure 18. Finally, lane 8 is the molecular weight markers.

Figure 20 is a Western blot assay performed to verify the identify of the rec-pEGF obtained by gel electrophoresis. The protein was transferred to an Immobilon membrane and analyzed with commercially available antibodies to mouse EGF. Panel A illustrates a coomassie stained gel of rec-pEGF and mouse EGF on a 1D-PAGE gel, and panel B illustrates the rec-pEGF and mouse EGF proteins which are recognized by rabbit anti-mouse EGF (Upstate Biotechnology, Incorporated) and detected which chemiluminescence. In both panels A and B, lane 1 is a molecular weight marker, lane 2 is 5.5 µg of rec-pEGF, and lane 3 is 0.25 µg mouse EGF.

In Figure 21, the bioactivity of EGF was tested in a 3T3 fibroblast proliferation assay. EGF was added to quiescent Swiss 3T3 fibroblast cells and stimulation of fibroblast proliferation was measured by $[H^3]$-thymidine incorporation as an indicator of DNA synthesis. Stimulation of fibroblast proliferation by rec-pEGF was comparable to that of purified mouse EGF.

Figure 22 is a graph comparing the average size of the ten largest follicles observed in histological sections of ovarian obtained from necropsy samples of the pigs in Group 1, above, comparing follicle size to that of the control group and the group administered nothing. Follicular diameter was measured using an ocular micrometer and only sections through the center of a follicle, having the germinal vesicle present, were used for the evaluation. It will be appreciated that the follicle size in the pigs given rec-pEGF were over 100% greater in diameter than that of the control group and over 70% greater than that of the group given nothing. Similarly, Table 7, below, offers a comparison of the organ weights of control and EGF-treated pigs in Group 2, and demonstrate the lack of effect of rec-pEGF on organ weights, body weights and general pathology.

TABLE 7

Measure	Control N=3	EGF N=3	Treatment P Value
Day 14 Weight (kg)	4.9 ± 0.24	4.6 ± 0.24	0.46
Day 70 Weight (kg)	24.5 ± 3.40	22.7 ±3.40	0.72
Avg. Daily Gain (kg/d)	0.35 ± 0.06	0.32 ± 0.06	0.76
Heart (gm)	124.4 ± 11.3	114.4 ± 11.3	0.56
Heart (% Body Weight)	0.52 ± 0.03	0.51 ± 0.03	0.91
Spleen (gm)	129.6 ± 38.1	112.0 ± 38.1	0.76
Spleen (% Body Weight)	0.53 ± 0.13	0.48 ± 0.13	0.82
Liver (gm)	703.9 ± 71.3	622.6 ± 71.3	0.50
Liver (% Body Weight)	2.91 ± 0.16	2.77 ± 0.16	0.57
Uterus (gm)	8.06 ± 0.82	8.15 ± 0.82	0.94
Uterus (% Body Weight)	0.034 ± 0.006	0.037 ± 0.006	0.77
Ovary (gm)	0.18 ± 0.085	0.36 ± 0.86	0.21
Ovary (% Body Weight)	0.0008 ± 0.0003	0.0018 ± 0.0003	0.23
Kidney (gm)	151.8 ± 18.1	139.8 ±18.12	0.81
Kidney (% Body Weight)	0.62 ± 0.07	0.64 ± 0.74	0.86

Finally, Figure 23 is a graph that confirms that *in vivo* administration of rec-pEGF to the Group 1 pigs, above, starting at 30 d.pp. and administered for fourteen consecutive days at a 540 μg/day dose by placement of subcutaneous osmotic pumps in each pig. Ovaries from a sub-set of Group 1 pigs were examined at 70 d.pp. when the first cohort of growing follicles is normally expected to be present. The data demonstrates clearly that the number of ovarian follicles were significantly increased by application of rec-pEGF over the control group and the group administered nothing. Data was obtained by counting the number of hematoxylin/eosin stained follicles in a counting frame (960 x 1400 μm), and only sections through the center of a follicle with the germinal vesicle present were used for the evaluation. Growing follicles were identified based upon the presence of at least one cuboidal layer of granulosa cells surrounding the oocyte.

The data clearly supports the proposition that administration of exogenous EGF accelerates ovarian development, increases ovulation rate at an age earlier than in untreated mammals, and increases the rate of ovarian maturation in prepubertal vertebrates. Additionally RNA samples were transferred to nylon membranes and will be evaluated for relative amounts of Z3α mRNA and will conform an increased level of follicular analysis by Northern blot analysis.

Although the invention has been described with respect to specific embodiments, it should be appreciated that other embodiments employing the concept of the present invention are possible without departing from the scope of the invention. The invention, for example, is not intended to be limited to the specific mammals discussed and exemplified and disclosed in these embodiments; rather the invention is defined by the claims in equivalence thereof.

What is claimed is:

1. A method for regulating vertebrate ovarian maturation and function, comprising the step of providing an amount of Epidermal Growth Factor to prepubertal ovaries of female vertebrate species sufficient to stimulate primordial ovarian follicular development and activate dormant ovarian follicles.

2. The method according to Claim 1, wherein the vertebrate species is swine.

3. A method for accelerating the onset of puberty in a prepubertal vertebrates, comprising the step of administering an amount of Epidermal Growth Factor sufficient to stimulate primordial ovarian follicular development and activate dormant ovarian follicles.

4. The method according to Claim 3 wherein the vertebrate are mammals.

5. The method according to Claim 4 wherein the mammals are selected from the group consisting of bovine, equine, porcine, canine, feline, human, farm animals and zoo animals.

6. The method according to Claim 1, wherein the vertebrate is swine and the Epidermal Growth Factor is administered to the swine less than or equal to 50 days post partum to activate ovarian and follicular development.

7. The method according to Claim 1, wherein the Epidermal Growth Factor activation of dormant follicles is monitored by gene markers which are initially transcribed in the activated primordial follicles and increase through stages of follicular development.

8. The method according to Claim 7, wherein the zona pellucida genes R55 and R75 are markers, which are initially transcribed in, activated primordial follicles.

9. The method according to Claim 1, wherein activation of dormant follicles and expression of the zona pellucida gene are increased by at least 200% by treatment of prepubertal mammals with Epidermal Growth Factor.

10. The method according to Claim 1, wherein a zona pellucida gene is a marker for determining whether Epidermal Growth Factor stimulates activation of primordial follicles, thereby resulting in expression of genes by oocytes in early stages of follicular development.

11. The method according to Claim 10, wherein the zona pellucida gene is ZP3-alpha and the expressed gene is a ZP gene.

12. A method for promoting ovulation in vertebrate species in species of different ages, comprising the step of providing an amount of Epidermal Growth Factor sufficient to initiate ovarian folliculogenesis.

13. The method according to Claim 12, wherein the species is the human female.

14. A method for effecting or regulating vertebrate ovarian maturation, comprising the step of administering a therapeutically effective amount of Epidermal Growth Factor to prepubertal vertebrates at different ages before primordial ovarian follicular maturation.

15. The method according to Claim 14, wherein the vertebrates are selected from the group of mammals comprising bovine, equine, porcine, canine, feline, human, farm animals and zoo animals.

16. The method according to Claim 14, wherein the vertebrate species is swine and the Epidermal Growth Factor is administered to the swine before fifty days post partum.

17. A method for promoting ovulation in vertebrate species, comprising the step of providing a therapeutically effective amount of Epidermal Growth Factor.

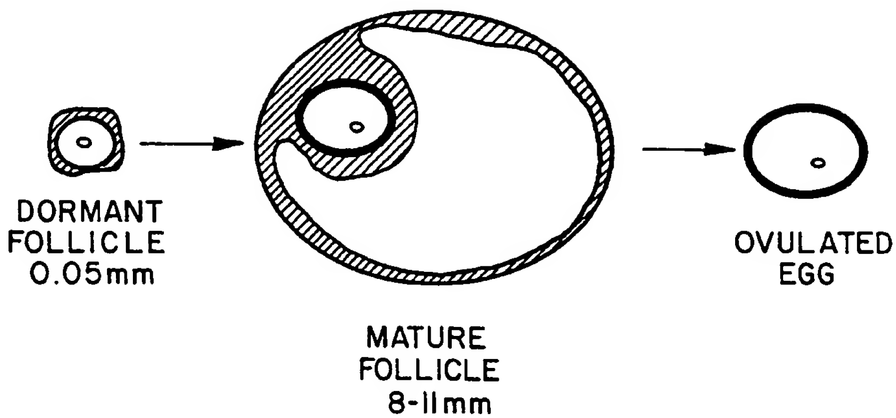

DORMANT
FOLLICLE
0.05mm
MATURE
FOLLICLE
8-11mm
OVULATED
EGG

Fig. 1

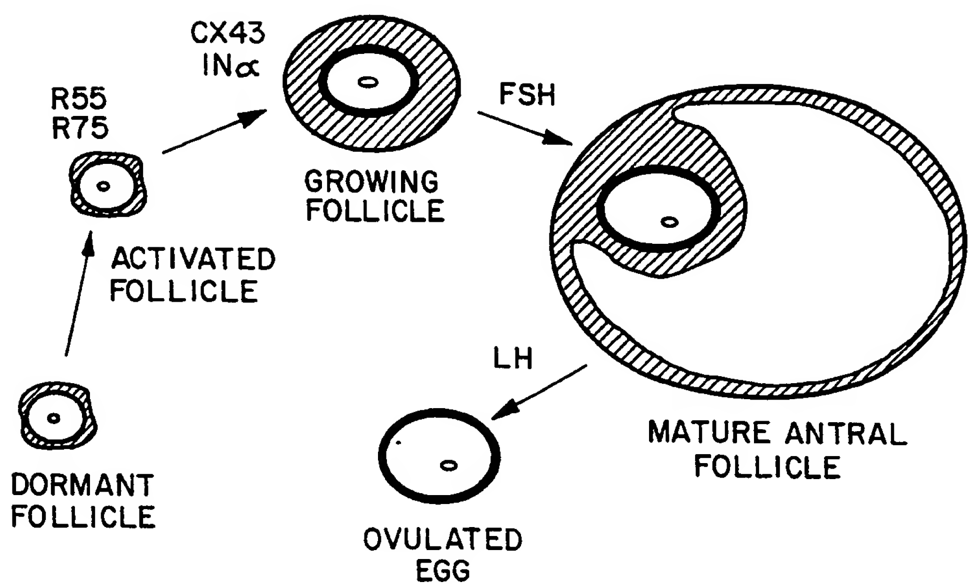

R55
R75
CX43
INα
FSH
GROWING
FOLLICLE
ACTIVATED
FOLLICLE
LH
DORMANT
FOLLICLE
MATURE ANTRAL
FOLLICLE
OVULATED
EGG

Fig. 9

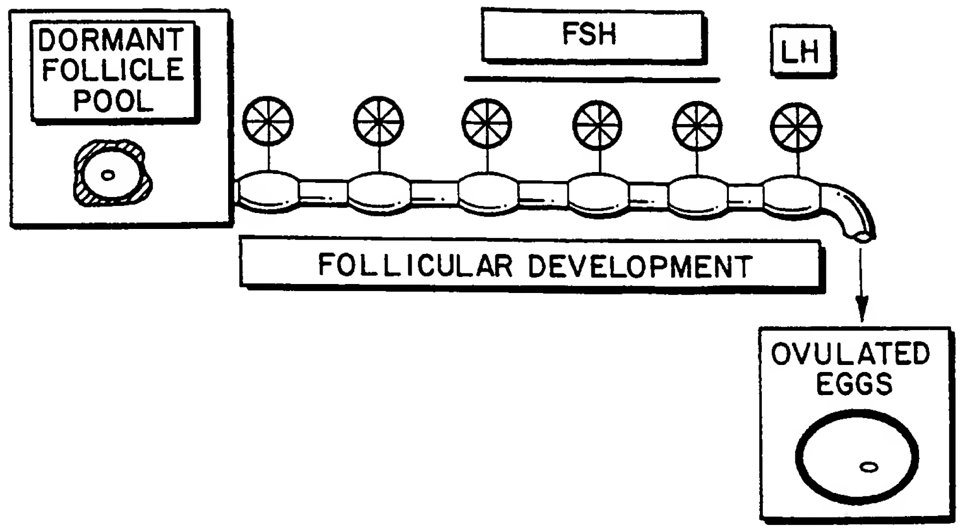

DORMANT
FOLLICLE
POOL
FSH
LH
FOLLICULAR DEVELOPMENT
OVULATED
EGGS

Fig. 2

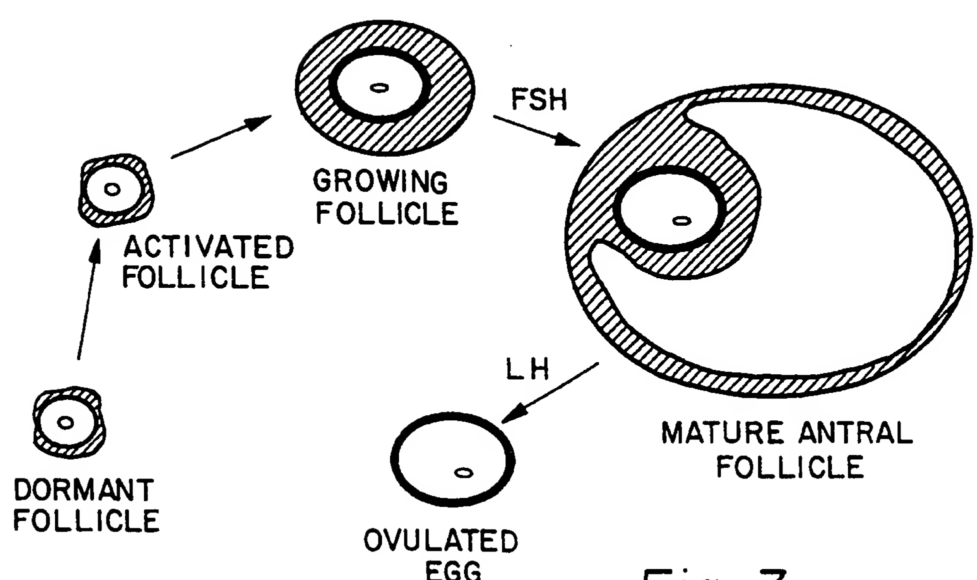

FSH
GROWING
FOLLICLE
ACTIVATED
FOLLICLE
LH
MATURE ANTRAL
FOLLICLE
DORMANT
FOLLICLE
OVULATED
EGG

Fig. 3

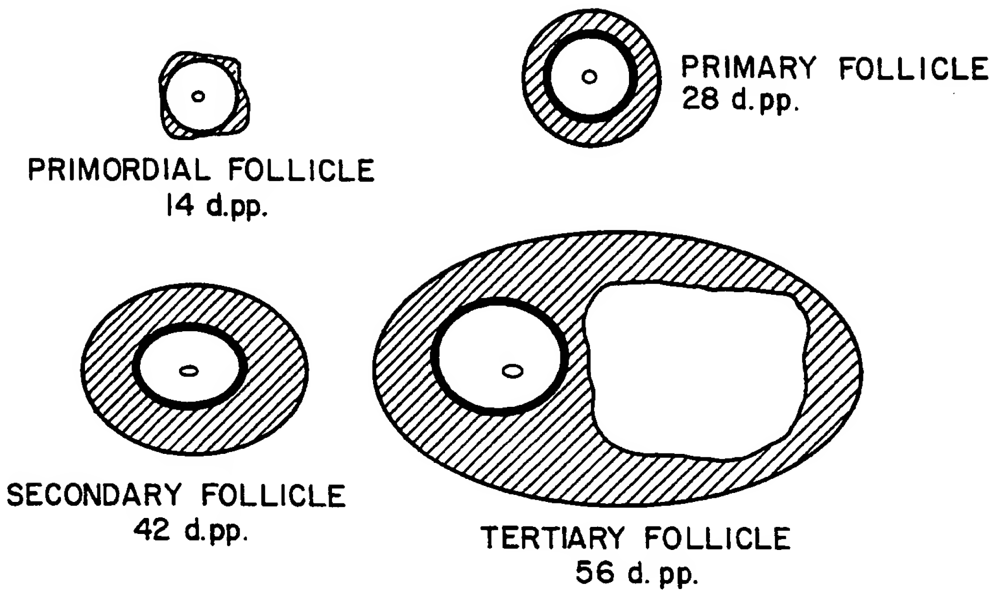

PRIMORDIAL FOLLICLE
14 d.pp.
PRIMARY FOLLICLE
28 d.pp.
SECONDARY FOLLICLE
42 d.pp.
TERTIARY FOLLICLE
56 d. pp.

Fig. 4

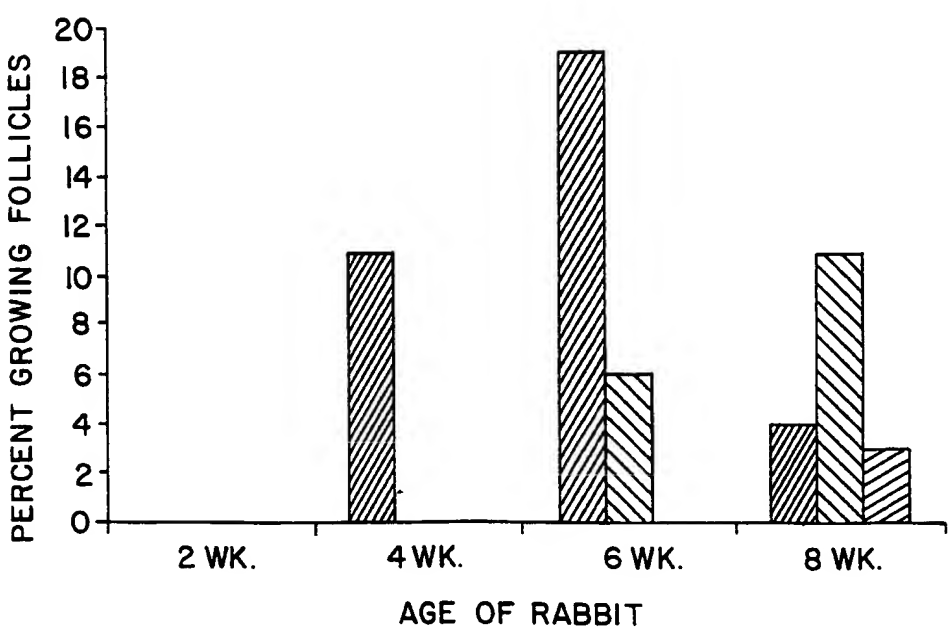

PERCENT GROWING FOLLICLES
20
18
16
14
12
10
8
6
4
2
0
2 WK.
4 WK.
6 WK.
8 WK.
AGE OF RABBIT

Fig. 5

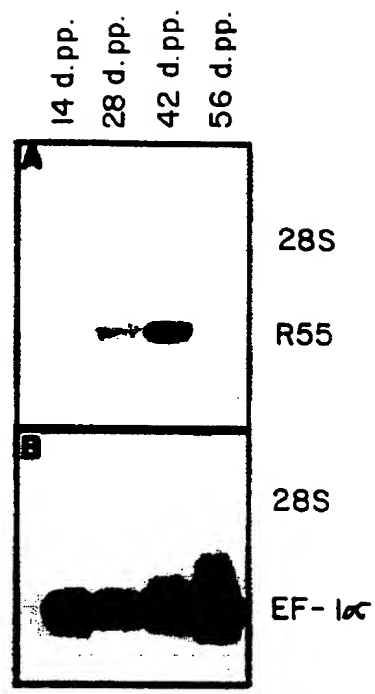

14 d.pp.
28 d.pp.
42 d.pp.
56 d.pp.
A
28S
R55
B
28S
EF-1α

Fig. 6

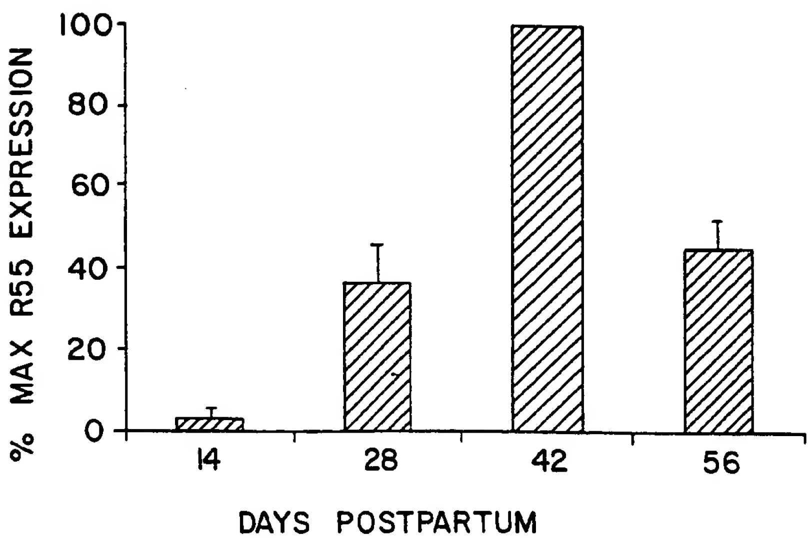

% MAX R55 EXPRESSION
100
80
60
40
20
0
14
28
42
56
DAYS POSTPARTUM

Fig. 7

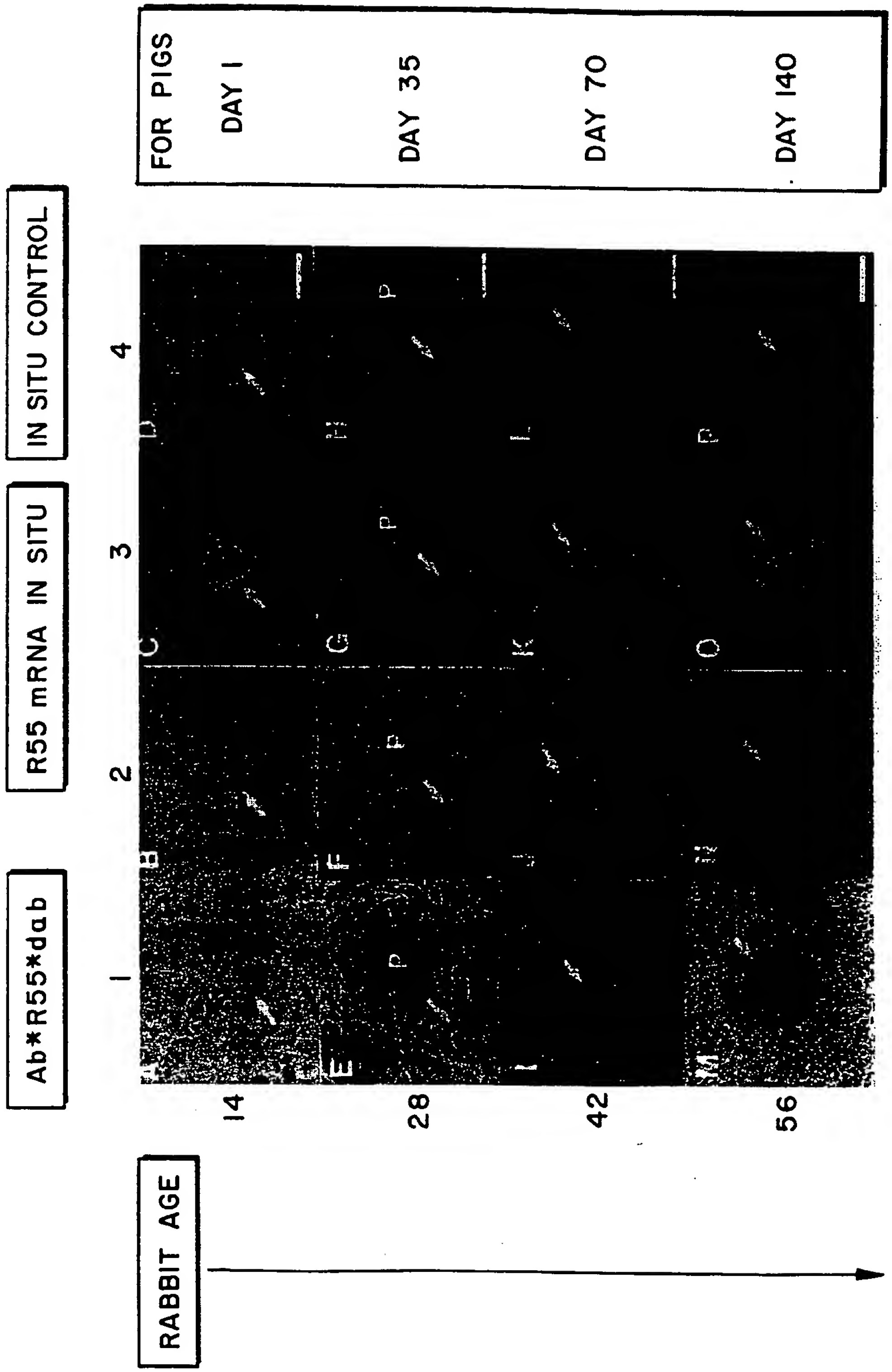

Ab*R55*dab
R55 mRNA IN SITU
IN SITU CONTROL
1
2
3
4
FOR PIGS
DAY 1
DAY 35
DAY 70
DAY 140
RABBIT AGE
14
28
42
56

Fig. 8

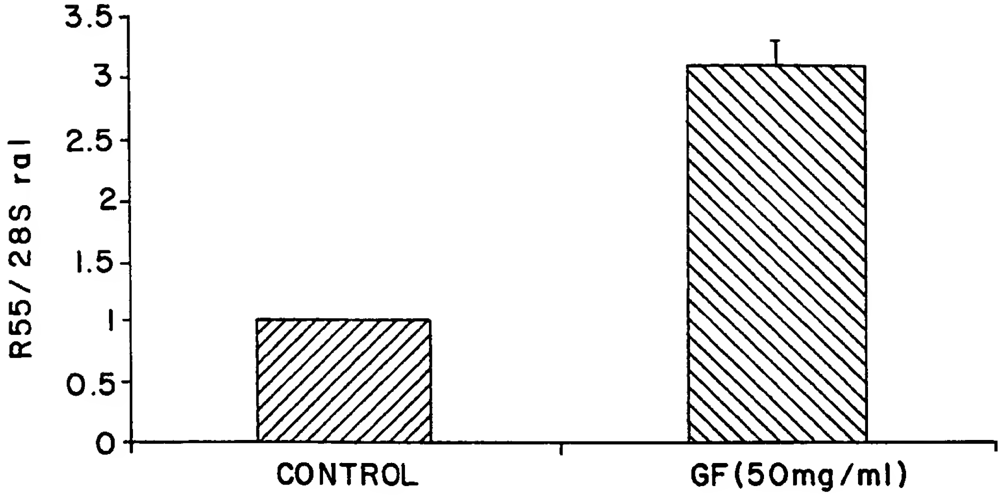

3.5
3
2.5
2
1.5
1
0.5
0
R55/28S ral
CONTROL
GF(50mg/ml)

Fig. 10

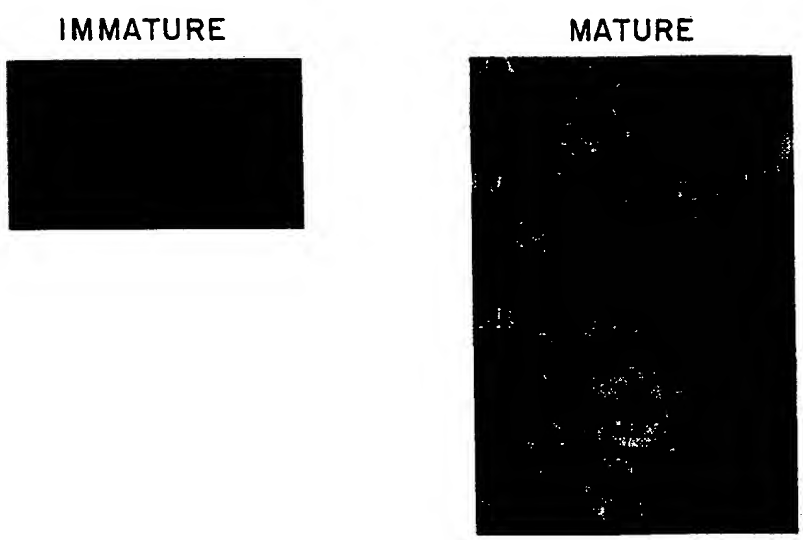

IMMATURE
MATURE

Fig. 11

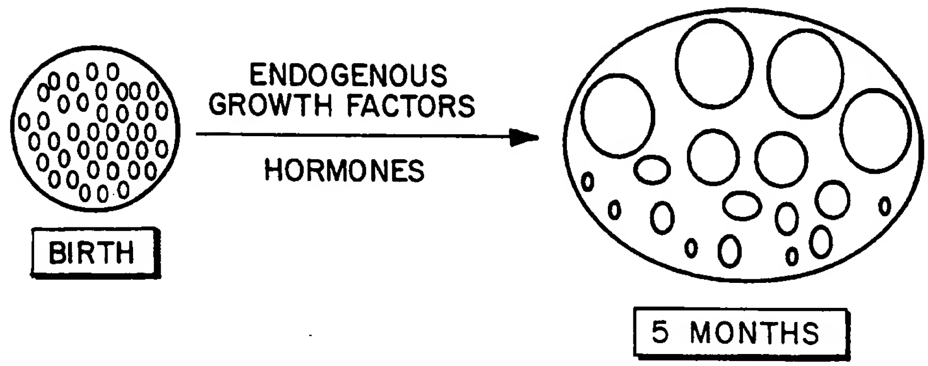

ENDOGENOUS
GROWTH FACTORS
HORMONES
BIRTH
5 MONTHS

Fig. 13

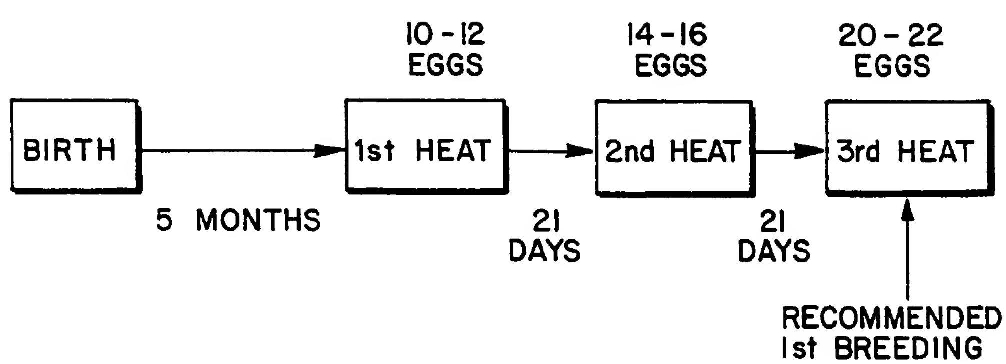

10-12
EGGS
14-16
EGGS
20-22
EGGS
BIRTH
1st HEAT
2nd HEAT
3rd HEAT
5 MONTHS
21
DAYS
21
DAYS
RECOMMENDED
1st BREEDING

Fig. 14

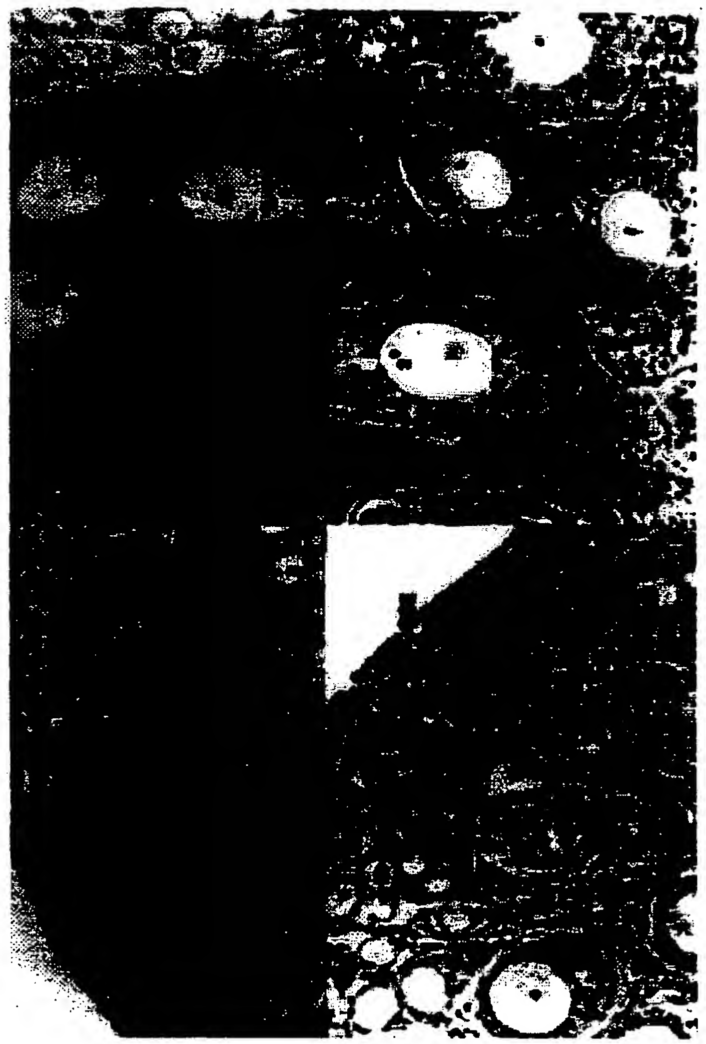


Fig. 12

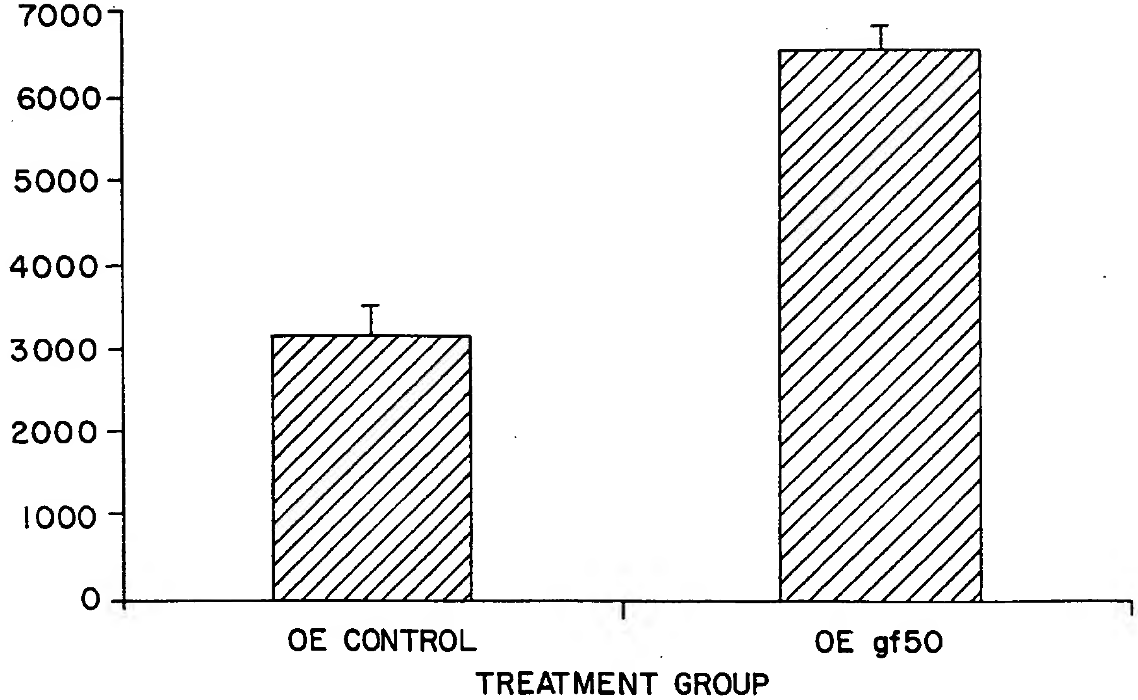

7000
6000
5000
4000
3000
2000
1000
0
OE CONTROL
OE gf50
TREATMENT GROUP

Fig. 15

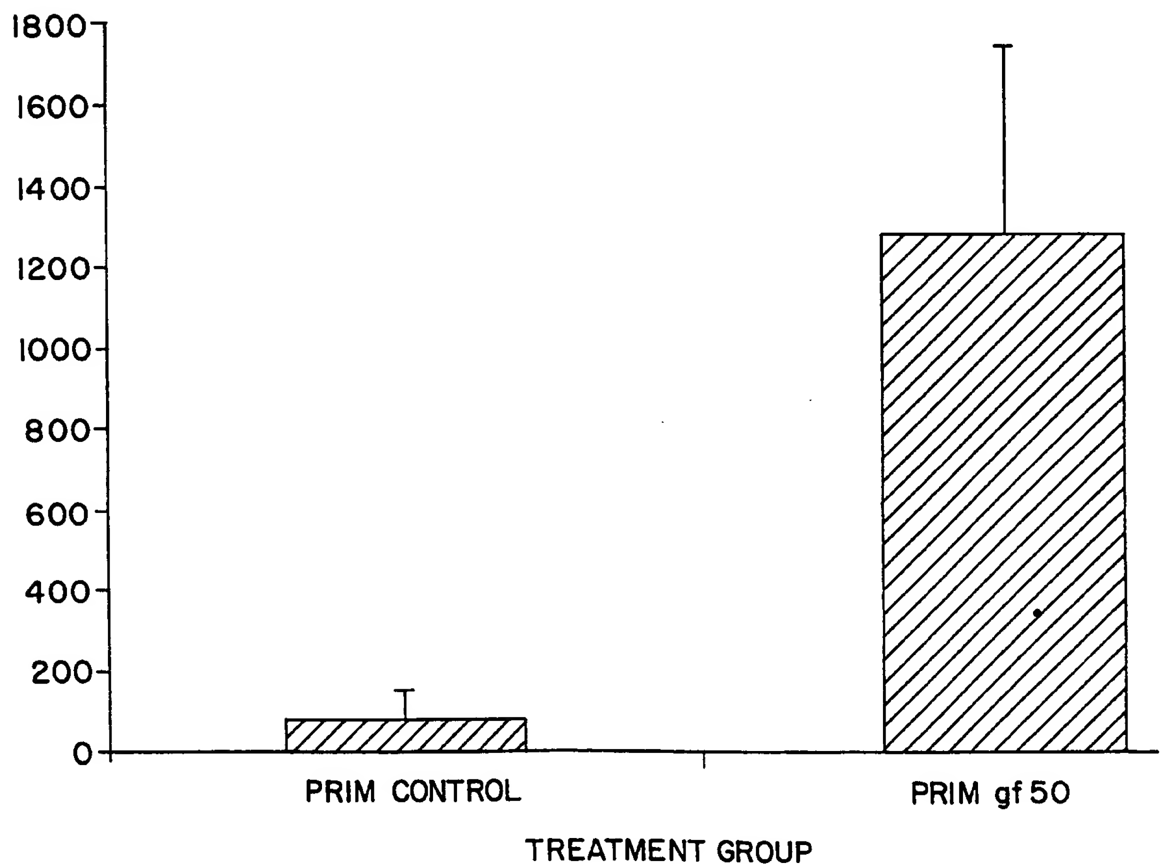

1800
1600
1400
1200
1000
800
600
400
200
0
PRIM CONTROL
PRIM gf50
TREATMENT GROUP

Fig. 16

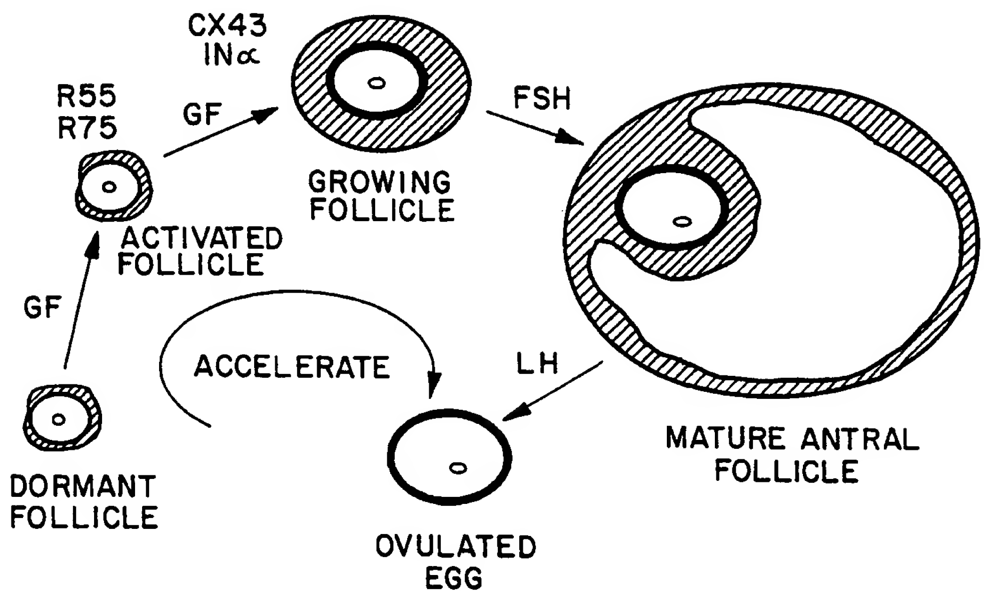
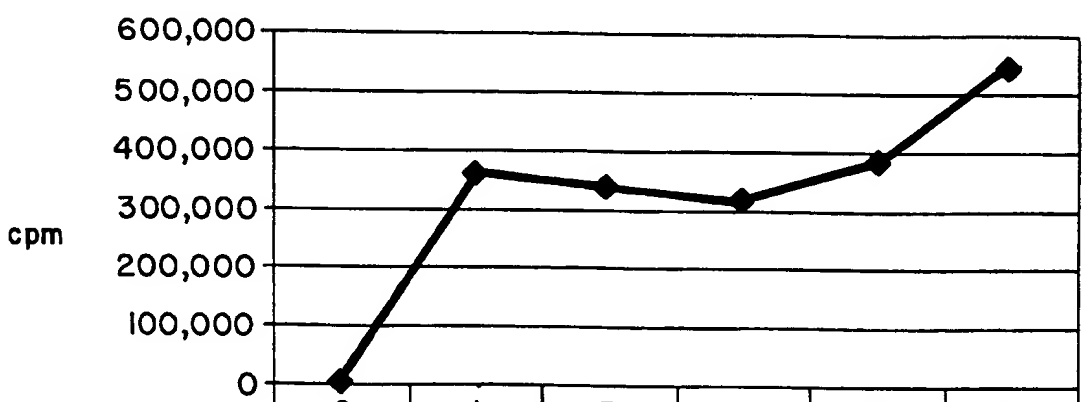


Fig. 17



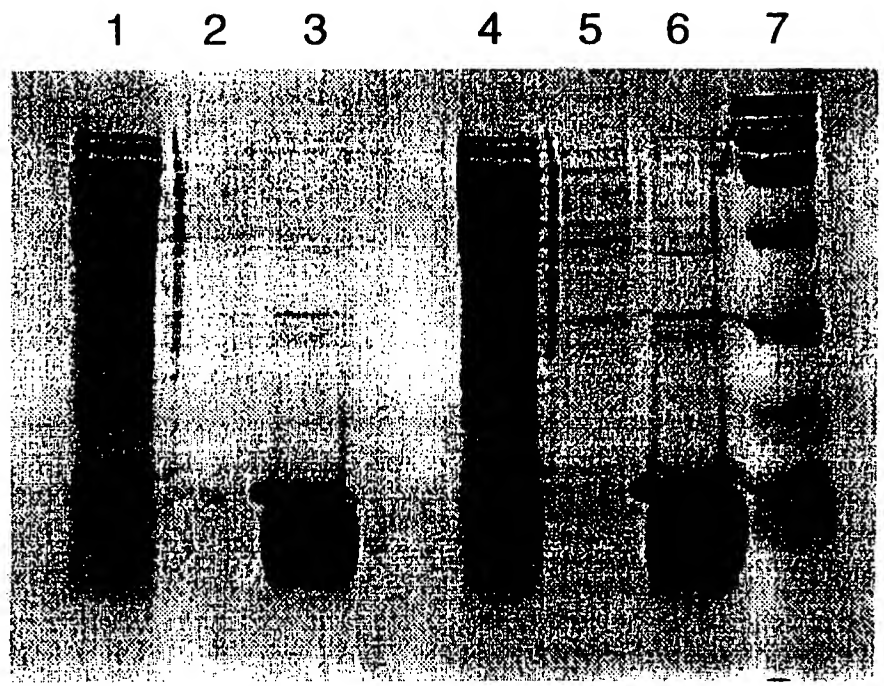
	0	1	5	10	50	100
cpm	1,994	359,352	339,806	318,333	384,735	550,857

pEGF, ng/mL

Fig. 21

```
         ATGAGAGGATCGCATCACCATCACCATCACGGATCCAATAGTTACTCTGAATGCCCGCCG
       1 ---------+---------+---------+---------+---------+---------+ 60
         TACTCTCCTAGCGTAGTGGTAGTGGTAGTGCCTAGGTTATCAATGAGACTTACGGGCGGC

a        M  R  G  S  H  H  H  H  H  H  G  S  N  S  Y  S  E  C  P  P  -

         TCCCACGACGGGTACTGCCTCCACGGTGGTGTGTGTATGTATATTGAAGCCGTCGACAGC
      61 ---------+---------+---------+---------+---------+---------+ 120
         AGGGTGCTGCCCATGACGGAGGTGCCACCACACACATACATATAACTTCGGCAGCTGTCG

a        S  H  D  G  Y  C  L  H  G  G  V  C  M  Y  I  E  A  V  D  S  -

         TATGCCTGCAACTGTGTTTTTGGCTACGTTGGCGAGCGATGTCAGCACAGAGACTTGAAA
     121 ---------+---------+---------+---------+---------+---------+ 180
         ATACGGACGTTGACACAAAAACCGATGCAACCGCTCGCTACAGTCGTGTCTCTGAACTTT

a        Y  A  C  N  C  V  F  G  Y  V  G  E  R  C  Q  H  R  D  L  K  -

         TGGTGGGAGCTGCGCAAGCCGAATTCGAGCTCGTACCCGGGGTCCTCTAGAGTTGACCTG
     181 ---------+---------+---------+---------+---------+---------+ 240
         ACCACCCTCGACGCGTTCGGCTTAAGCTCGAGCATGGGCCCCAGGAGATCTCAACTGGAC

a        W  W  E  L  R  K  P  N  S  S  S  Y  P  G  S  S  R  V  D  L  -

         CAGCCAAGCCGATAG
     241 ---------+----- 255
         GTCGGTTCGGCTATC

a        Q  P  S  R  *  -
```

Fig. 18



Lane 1: LP6A-SP1 Flow Through (unbound bacterial proteins).
Lane 2: LP6A-SP1 Buffer Wash of column.
Lane 3: LP6A-SP1 Eluted rec-pEGF from column.
Lane 4: LP6B-SP1 Flow Through (unbound bacterial proteins).
Lane 5: LP6B-SP1 Buffer Wash of column.
Lane 6: LP6B-SP1 Eluted rec-pEGF from column.
Lane 7: Rainbow Molecular Weight Markers.

Fig. 19

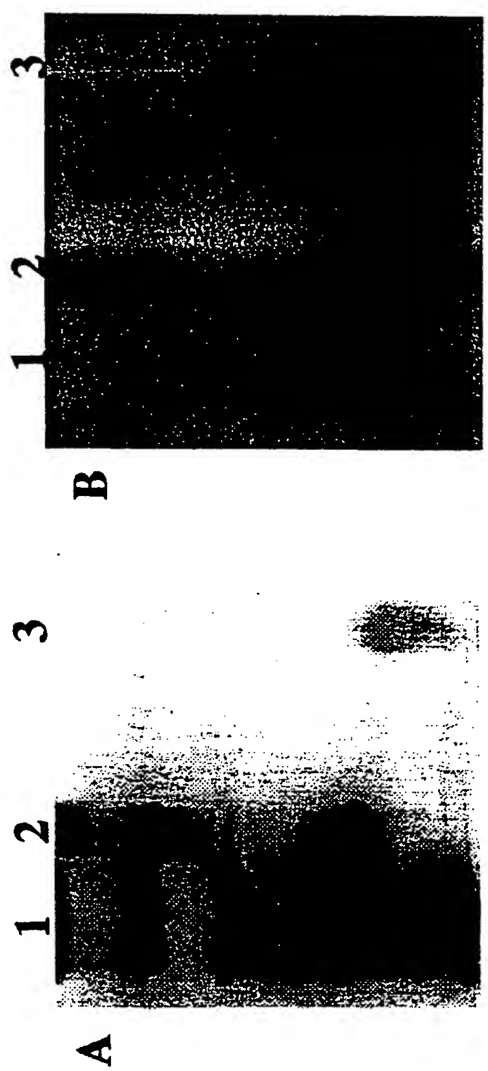


Fig. 20

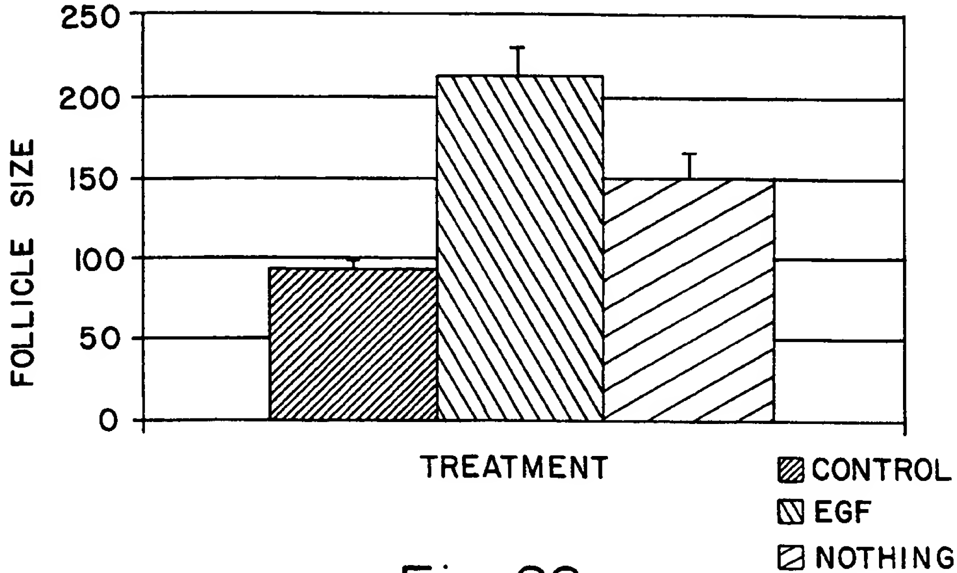

250
200
150
100
50
0
FOLLICLE SIZE
TREATMENT
CONTROL
EGF
NOTHING

Fig. 22

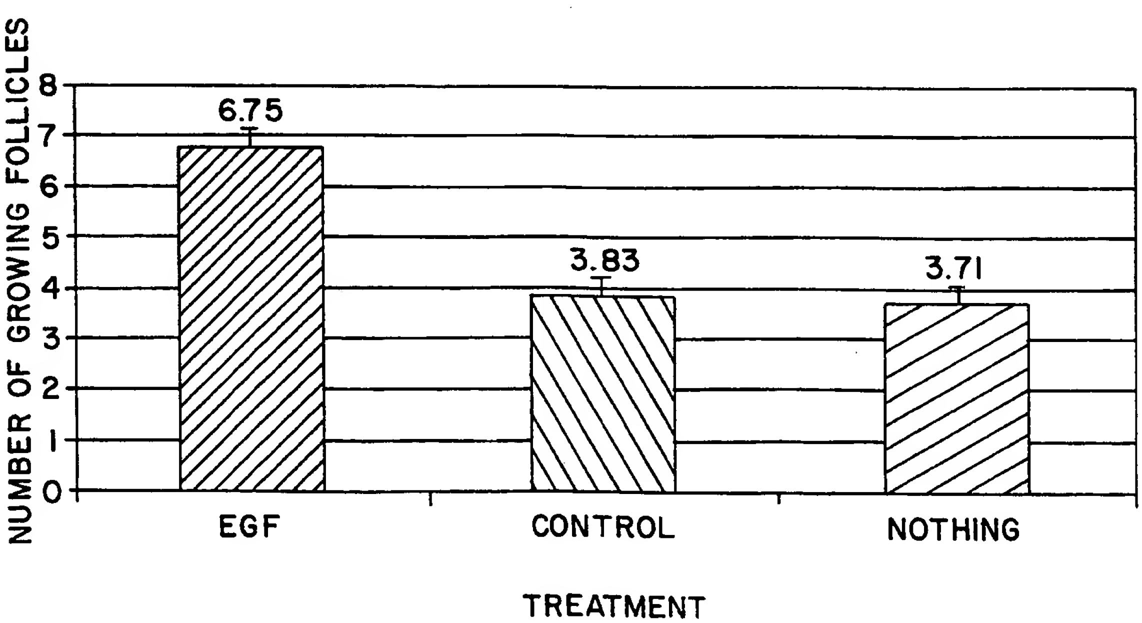

8
7
6
5
4
3
2
1
0
NUMBER OF GROWING FOLLICLES
6.75
3.83
3.71
EGF
CONTROL
NOTHING
TREATMENT

Fig. 23

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(54) Title: REGULATION OF OVARIAN MATURATION AND FUNCTION USING EPIDERMAL GROWTH FACTOR

(57) Abstract

A method is provided for regulating vertebrate ovarian maturation and function using growth factors by providing an amount of epidermal growth factor to prepubertal ovaries of female vertebrate species including stimulation of primordial follicles and enhancing activation of dormant follicles with the results that the mechanics of the method regulate early development of ovarian follicles. The method provides activation of dormant follicles and early ovarian maturation which is accelerated to the point of producing earlier breeding cycles as well as increasing first litter sizes. The method for regulating vertebrate/mammal ovulation maturation is also directed to increasing the ovulation rate and increasing litter size at an age of normal breeding.

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REGULATION OF OVARIAN MATURATION AND FUNCTION USING EPIDERMAL GROWTH FACTOR

Field of the Invention

The present invention relates generally to methods of regulating ovarian follicular development, maturation and ovulation to facilitate vertebrate mammalian reproduction. More specifically, the present invention relates to a method whereby Epidermal Growth Factor ("EGF") is administered to a vertebrate mammal to promote ovarian follicular development, maturation and ovulation and/or accelerate follicular differentiation in the preantral stages and increase the number of ova available for fertilization at each reproductive cycle. In another aspect, the invention relates to the treatment of prepubertal mammals and vertebrate subjects with EGF to regulate and accelerate ovarian maturation and function.

Background of the Invention

Many diagnostic and therapeutic procedures exist to aid reproduction practitioners in reproductive diagnostics, therapeutics and interventions. In mammalian embryology, mammalian oocytes enter the first meiotic division during fetal life, but become arrested in late prophase (in the dictate or diffuse diploid stage of meiosis) before or just after birth (Beaumont, H.M., et al., *Proc. R. Soc. London* (Series Biological Sciences) 155:557-579 (1962). Resumption of meiosis normally does not occur until shortly before the first ovulation, when previously unidentified growth factor trigger ovarian development, followed by a surge of gonadotropins prompts the resumption of meiotic maturation (Dekel, N., et al., *Proc. Nat'l Acad. Sci. U.S.A.*, 75:4369-4373 (1978).

Currently, infertility in humans ranges from approximately 10-15% of couples and the risk of infertility is doubled for women between the ages of 35-44 when compared to women between the ages of 30-34. In the United States, the majority of infertility can be accounted for by reproductive problems in the female.

In U.S. Patent No. 5,395,825, investigators provided methods for determining swine ovarian follicular developmental stages. Laminin is a basement membrane protein that must be synthesized and secreted as the follicle grows. Thus, the development of laminin may be used as a specific for granulosa cell differentiation and development in early stages of follicular growth. In another study, published by Vinter-Jensen et al., in 1995, EGF

administered to mini-pigs was found to stimulate growth of heart, liver, and urinary tract tissue. Reproductive organs, however, were not closely evaluated in any of these studies. Eppig and O'Brien published data in 1996 which showed that EGF treatment of neonatal mouse ovaries, cultured *in vitro*, yielded an increased the number of eggs recovered for *in vitro* maturation and fertilization. This data also implicates a role for EGF in follicular development. Breider, *et al.*, studied the effect of intravenous infusion of EGF into mature rats and found that EGF stimulated growth and proliferation of may different types of tissues, including the mature rat ovaries, which exhibited increased ovarian weight, accompanied by the increase in the number of corpora lutea found in these ovaries. The corpora lutea is a structure that results from the ovulation of a mature antral follicle. Thus, in the mature rat, there is evidence that EGF administration increases the number of mature ovulations. Again, these studies were done in live animals suggesting the feasibility of using EGF *in vivo*.

Epidermal Growth Factor is a peptide hormone that stimulates the growth and differentiating of epidermal tissues during embryogenesis (Carpenter, G., et al., *Exper. Cell. Res*. 164-1-10 (1986); Kris R. M. et al., *Bio-Technol.* 3:135-140 (1985)). EGF may be purified from natural sources or may be obtained through application of recombinant DNA technology. EGF is a 53-residue polypeptide (M, -6000) that is mitogenic for a variety of cell types both in vivo and in vitro (Carpenter & Cohen, 1979). EGF was originally purified from the male mouse submaxillary gland (SMG) (Cohen 1962) and subsequently from human urine (Cohen & Carpenter, 1975; Gregory, 1975). Antibodies raised against mouse or human EGF are used to confirm expression in tissues or body fluids using immunoassays or immunocytochemical staining. Highest levels of EGF have been found in SMG (mouse), kidney, pancreas, duodenum, urine and milk (see Carpenter, 1985; Gregory, 1985; Burgess, 1989; Fisher & Lakshmanan, 1990). However, there is little information regarding EGF expression in other species because antisera against EGF show very little cross-species reactivity (Gregory, Holmes & Willshire, 1979; Schaudies & Savage. 1986) necessitating the development of homologous immunoassays (Joh. Itoh, Yasue et al. 1989). Despite their limited immunological cross-reactivity, both mouse and human EGF bind to cellular receptors on various cell types from several species with very similar affinities and efficacy (see Carpenter & Cohen, 1979; Carpenter, 1987). While heterologous radioreceptor assays are therefore possible, they lack specificity since other polypeptides (*e.g.* transforming growth factor alpha ("TGFα") are known to bind to the same receptors (see Burgess, 1989;

Massague, 1990). In addition, indirect modulation of EGF receptor affinity by heterologous ligands has been widely reported (see Schlessinger. 1986).

A homologous radioimmunoassay for the measurement of EGF levels in pig tissues and body fluids has been developed using an antiserum to recombinant porcine EGF. The assay is highly specific, showing no cross-reactivity with a variety of other polypeptides including the structurally related protein, transforming growth factor-alpha ("TGFα"). Furthermore, less than one- percent cross-reactivity was observed with mouse EGF emphasizing the necessity for homologous assays for EGF measurement. immunoreactive EGF was present in extracts of pig kidney and pancreas (3.44 +1- 0.43 and 0.76 $\pm$ 0.13 (S.E.M.) pmol/g wet weight respectively), but was not detected in extracts of submaxillary gland or liver. Although immunoreactive EGF was not detectable in uterine, allantoic or ovarian follicular fluids, colostrum contained EGF at biologically active concentrations. Immunoreactive EGF was also present in pig urine, with similar concentrations in samples from male or female animals. In addition, pig urine inhibited the binding of iodine-labeled EGF to 3T3 fibroblasts and stimulated DNA synthesis in quiescent monolayers of these cells, indicating that the immunoreactive material in urine is biologically active. Quantitative comparisons of the data presented here with that published previously indicate considerable species variation in the EGF levels of various tissues and body fluids.

In follicular development, the development of a dormant primordial follicle into a large mature follicle must occur before the ultimate mature follicle is stimulated to ovulate and produce ova capable of fertilization. This general process is the key for the production of eggs in many species. Thus, the ovary functions as a reservoir of dormant follicles and through the process of follicular development, some of these dormant follicles will develop and mature to produce hundreds to thousands of eggs. Follicular development can be envisioned as a pipeline and the control of the process occurs by many regulatory steps. In the later stages of follicular development, it is known that Follicle Stimulating Hormone ("FSH") is important for growth and development of mature antral follicles and ultimately Lutenizing Hormone ("LH") stimulates the ovulation of a mature follicle and the production of the egg. However, the factors regulating early steps of follicular development have been unknown.

The present invention provides methods to influence the early regulatory steps that control ovarian follicular development in vertebrate and mammalian species. The present

invention, may, for example, be utilized in bovine, equine, porcine, canine, feline, human mammals, avian, aquatic, reptilian species or the like. It is contemplated that the present invention will directly benefit the production of food species, such as bovine, swine, and avian and aquatic food species, such as chicken, turkey, duck, salmon, cod, trout or the like, in other farm animals, assist in human infertility, and in reproduction of endangered species and zoo animals.

Summary of the Invention

The present invention is directed to a method of initiating and regulating ovarian follicular development in mammalian females. In the present invention, rabbits and pigs were used as vertebrate mammalian models. Genetic markers have been identified which are initially transcribed in activated primordial follicles in rabbits and pigs. Expression of two rabbit zona pellucida genes R55 and R75, were localized in prepubertal rabbit ovaries by in situ hybridization. Results indicate that transcription of the genes occur initially in activated follicles and increases through early stages of follicular development. Expression of the R55 and/or R75 genes offers a qualitative method to identify activated follicles *in vivo* and a quantitative method to activate follicular development *in vitro*.

In addition to permitting identification of morphological changes and granulosa cell proliferation, these gene markers provide functional definitions for the initial steps in folliculogenesis. To determine whether growth factors can stimulate activation of primordial follicles, tissue explants from immature rabbit ovaries (14 days old) were cultured with or without mouse EGF. Ovaries at this age contain only primordial follicles providing an ideal population in which to study activation of follicular development. The relative amounts of R55 mRNA were measured by Northern blot analysis. The Northern blot assay results indicated that EGF (50 ng/ml) increased expression of R55 in primordial rabbit ovarian follicles. It was concluded that EGF stimulates expression of zona pellucida genes in primordial follicles and enhances the level of activation of dormant follicles.

The ability to regulate both the timing and the magnitude of follicle activation can influence the overall reproductive capacity of a given female and lead to new methods for managing reproductive function in clinical or agricultural settings.

In another aspect, the present invention is directed toward using EGF in swine to regulate ovarian follicular development, ovarian maturation and ovulation. Swine production is limited, in part, because during early breeding cycles relatively few ova are ovulated,

resulting in small litter sizes in young sows. Thousands of ovarian follicles containing eggs are formed in new born animals and are available to be activated, but for unknown reasons only a few mature in early estrus cycles. By accelerating earlier activation of dormant ovarian follicles and earlier ova maturation, the present invention provides a method for enhancing productive efficiency in sows accelerating breeding cycles and increasing earlier litter sizes.

Rabbits have ovarian development patterns similar to pigs. The rabbit model was employed to model swine ovarian development. Rabbit test results indicate that activation of dormant follicles and expression of the rabbit R55 zona pellucida gene are increased approximately three fold by treatment of prepubertal ovaries with EGF. Pig follicles synthesize a zona pellucida molecule, ZP3α, which is 74% identical to rabbit R55 and is detectable with molecular probes to R55. This molecular marker for activation and techniques established in the rabbit model was used for studies with pig ovaries.

Confirming these findings in two distinct mammalian species, namely rabbit and swine, suggests a commonality to all vertebrates and mammals that the mechanism of early follicular activation may be accelerated by administering EGF.

Brief Description of the Figures

Fig. 1 is a diagram showing a generalized overview of follicular development;

Fig. 2 is a schematic diagram presenting the many potential steps and control points for follicular development;

Fig. 3 is a diagram showing the later stages of follicular development which occur at growing follicles when they respond to stimulation by FSH and where they mature into antral follicles;

Fig. 4 is a diagram that shows the different stages of follicular development that are present in prepubertal rabbits;

Fig. 5 is a graph demonstrating the first wave of follicular development in prepubertal rabbits;

Fig. 6 is a photograph representation showing the Northern blot analysis of R55 in the immature rabbit ovaries;

Fig. 7 is a graph showing R55 expression in developing rabbit ovaries;

Fig. 8 is a composite representation showing the localization of R55 protein and messenger RNA in developing ovaries of prepubertal rabbits;

Fig. 9 is a diagram showing that a second ZP gene in the rabbit R75 is a valid marker for activation of primordial follicles and its pattern of expression which is similar to R55;

Fig. 10 is a graph showing the effects of growth factors (epidermal growth factor, EGF) on R55 expression in immature rabbit ovaries;

Fig. 11 is a representation to ovarian development in prepubertal pigs;

Fig. 12 presents the follicle populations present in the immature pig ovaries at day 70 postpartum or 10 weeks of age.

Fig. 13 is a schematic depicting the follicle development that occurs during ovarian maturation in the prepubertal pig;

Fig. 14 presents a schematic that shows the reproductive maturation timeline as relative to the situation found with commercial gilts;

Fig. 15 is a graph showing the inventive results established in the rabbit was applicable to the pig;

Fig. 16 is a graph showing the effect of EGF on ZP expression in isolated primordial porcine or pig follicles.

Fig. 17 is a diagram summarizing overall basic working model for acceleration of follicular development in accordance with the invention.

Fig. 18 is the DNA and amino acid sequences of the recombinant porcine EGF (rec-pEGF) protein, which is expressed, in the bacterial system.

Fig. 19 is a representative sample of 15% polyacrylamide SDS gels of expressed rec-pEGF protein purified in Ni-Agarose columns.

Fig. 20 are Western blots of rec-pEGF, in which panel A is a coommassie stained 1D-PAGE gel and panel B is mouse EGF antibodies detected with chemiluminescence.

Fig. 21 is a graph of a fibroblast proliferation assay using tritiated thymidine uptake to determine the effect of increasing rec-pEGF concentrations on DNA synthesis.

Fig. 22 is a graph depicting the average size of the ten largest follicles observed in H/E-stained sections of pig ovaries, comparing the EGF administered group, with a control group and a group administered neither the EGF or a placebo.

Fig. 23 is a graph illustrating the number of growing ovarian follicles at 70 days of age for the EGF administered group, a control group and a group administered nothing.

<u>Detailed Description of the Invention</u>

Swine sows are polyestrous with a period of lactational anestrus occurring until after weaning. The estrus cycle length averages 21 days. Return to estrus occurs after the recovery period, or 4-7 days post-weaning. Estrus onset is marked by the preovulatory surge of LH. Gilts come in to first estrus around eight months of age. Estrus (heat behavior) occurs for 2-3 days, averaging 60 hours in mature sows, but is only about 48 hours long in gilts. Ovulation actually occurs during the last third of estrus.

Ovulation occurs 36 to 42 hours after the onset of standing heat in mature sows, about 12 hours earlier in gilts. On average, 10-20 ova are shed from follicles ranging from 0.7 to 1.0 cm. Fecundity is highest in sows at 2 to 4 years of age. Gilts average 10-15 ova per cycle. Embryonic survival rate is 65%-75%, resulting in litter sizes of 8 to 12 piglets. Heritability of litter size is low, but tremendous breed differences exist. Recent studies suggest fecundity may be linked to the presence of a specific estrogen receptor. In addition, fecundity is strongly linked to lactational feed intake. Limiting feed intake in gestation leads to greater feed intake during lactation. High feed intake in lactation results in good milk production, high piglet weights at weaning, short wean to estrus intervals, and maximal ovulation rates.

The zona pellucida (ZP) matrix surrounding oocytes appears in follicles following activation, indicating transcription of ZP genes is specifically development. The protein and mRNAs for the rabbit 55 kD ZP component, R55, are initially expressed in oocytes of activated follicles before morphological changes. Preliminary results indicated that pig ZP proteins are expressed in activated follicles and show that expression of the pig homologue to R55, ZP3α, provides a marker for determining initiation of follicular development in this species. An objective of the present invention is to determine that EGF stimulates activation of pig primordial follicles, subsequently resulting in expression of ZP genes by oocytes and accelerates the early stages of follicular development. Thus, ZP markers can provide an objective method for measuring follicle activation and development in addition to more subjective observations based on morphological changes. Furthermore, these experiments are necessary to determine effectiveness for using EGF to accelerate prepubertal ovarian development in neonatal pigs and ultimately increase reproductive efficiency. An increase of even 10% of pigs per litter would be economically significant for commercial pork producers.

Before follicular development begins, ovarian follicles are termed "primordial" or "dormant" follicles and consist of undifferentiated squamous granulosa cells surrounding an oocyte arrested in prophase I of meiosis. When activated, the granulosa cells proliferate and

differentiate, while the oocytes enter a growth phase. During these early steps of follicular development ZP proteins (rabbit R55 and pig ZP3α) are synthesized and assembled around the oocyte. Recent studies have begun to use culture systems to study regulation of these early stages of folliculogenesis. In preliminary observations R55 sense (+) and antisense (-) RNA probes were labeled with [35^{s}]-UTP and used for *in situ* hybridization to determine the spatio-temporal pattern of expression of R55 during early rabbit ovarian development. Ovaries were collected from prepubertal rabbits (14 and 28 days postpartum, d.pp.), fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for localization of R55.

Localization of R55 protein and mRNA in activated and growing follicles of prepubertal rabbit ovaries was demonstrated. In 14 d.pp. ovaries, R55 protein and mRNA were localized in oocytes of some primordial follicles but are undetectable in the majority of primordial follicles. It was illustrated that R55 protein and mRNA are abundant in oocytes of primary follicles from 28 d.pp. animals. Expression of R55 was incre*ased* in transitional follicles that exhibited characteristics of both primordial and primary follicles. In the cortex of ovaries from 28 d.pp. animals, many of the primordial follicles closest to the medullar region of the ovary are expressing R55 while it is undetectable in the cortical follicles. The position of these labeled primordial follicles indicated that they are the activated group of follicles that will develop during the peripubertal period of folliculogenesis. This spatio-temporal pattern of expression for R55 indicates that transcription of this gene is initiated in oocytes during the activation of follicular development.

Rabbit Ovary Culture

A method to culture pieces of ovaries from 14 d.pp. rabbits was developed, modeled after techniques used for ovaries from neonatal mice and bovine embryos. Tissue pieces (1-2 mm^3) from 14 d.pp. rabbit ovaries containing only primordial follicles were placed in cell culture inserts with 3.0 mm pores and cultured in 24-well plates with 300 microliters medium (50-50 blend of (a) F-12 Nutrient Mixture (Ham)(1X), liquid contains L-glutamine, and (b) Medium 199 (lX), liquid contains Earle's modified salts, 1.250 mg/L sodium bicarbonate, and L-glutamine.

After fourteen days post-treatment, the Group 1 pigs were necropsied and histological evaluation of ovarian sections (hematoxylin/eosin stained) revealed larger ovarian follicles and the presence of a 70% greater population of growing follicles in the rec-pEGF treated animals than in the control or non-treated groups.

The Group 2 pigs were grown to 10 weeks of age, then necropsied. At the time of necropsy, the pigs receiving rec-pEGF showed no negative effect on weight gain or organ development. The ovaries of the Group 2 pigs showed an almost 100% increase in size when the tissues were collected at 10 weeks of age, when compared to the control group and the group treated with nothing. Figures 22 and 23 set forth the foregoing results of the Group 1 and Group 2 pigs.

All pigs in Group 1 and in Group 2 were dosed using subcutaneously placed osmotic pumps. During the time of rec-pEGF, both groups were individually housed, with general health, feeding and behavioral observations made regularly and recorded.

Summary of In Vitro and In Vivo Results

The foregoing tests demonstrate that EGF stimulates expression of ZP3α and activates primordial follicles *in vitro* and in whole ovarian cultures. EGF stimulates activation of dormant pig follicles. EGF (50 ng/ml) stimulates DNA synthesis in granulosa cells from primary pig follicles and expression of FSH receptors in granulosa cells from antral pig follicles.

In pigs, an activated primordial follicle matures to antral stage in approximately 84 days and from activation to ovulation will be about 100 days. Therefore, normally follicles that ovulate in the first cycle were activated around day 50 post partum. The process can be accelerated (as we have shown) by application of EGF before day 50 post partum to activate ovarian and follicular development. The inventive goal is to increase the number of primordial follicles activated earlier in prepubertal development, thus increasing the number of growing follicles. Subsequent treatment with gonadotropins will result in more mature follicles at earlier ages in young sows. Further development of this model should result in larger and earlier litters in production sows.

Experiments on pigs demonstrate that pig ZP3α is expressed during activation of dormant primordial follicles and determines the period of prepubertal ovarian development during which follicle activation is greatest. In prepubertal pig ovaries, dormant primordial

Rabbit Ovary Cultures: Treatment With EGF

Cultures were treated with mouse EGF (50 ng/ml) and analyzed for the level of expression of R55 mRNA. Treatment with EGF produced a 3-fold increase in amount of R55 mRNA (normalized to 28S) as compared to controls incubated in medium alone (Figure 3). These results suggest that EGF stimulates activation of follicular development and expression of R55 in dormant primordial follicles.

Swine Ovary Cultures: Treatment With EGF

For *in vitro* experiments, ovaries from prepubertal pigs (3-5 weeks post partum) were collected for tissue cultures of ovarian pieces. The ovaries contained a large population of dormant primordial follicles similar to 14 d.pp. rabbits. Pig ovarian tissue cultures were treated with EGF at different doses for 6 days and tissues collected for *in situ* hybridization and Northern blot analysis of ZP3α. The 6 day culture period was chosen because in preliminary experiments with cultures of rabbit ovarian tissue, treatment with EGF (50 ng/ml) produced significant increases in expression of R55.

In Vivo EGF: Swine

For *in vivo* experiments, prepubertal pigs at different ages (1, 35, and 70 d.pp.) were treated with subcutaneous osmotic pumps delivering constant daily dosages of EGF for fourteen days. Ovaries were removed following treatments to evaluate follicle development. In ovarian tissue from *in vitro* and *in vivo* experiments, expression of ZP3α mRNA was localized by *in situ* hybridization and the percentage of activated primordial follicles determined. The amount of ZP3α mRNA in cultured pig ovarian tissue was determined by Northern blot analysis to quantitate the level of ZP expression (normalized to levels of 28S RNA).

Two groups of young female pigs were administered recombinant porcine EGF (rec-pEGF). Group 1, aged 30 d.pp., were given 540 µg/day rec-pEGF using subcutaneous osmotic pumps, for fourteen days. A control group of the same age were administered the injection vehicle only. A non-treated group was maintained under identical conditions as the control group and Group 1. Group 2, aged 21 d.pp., were given 600 µg/day rec-pEGF for fourteen days.

follicles are located in the outer portion of the cortex while activated and growing follicles are found close to the medullar region. Since ZP3α is expressed in activated follicles, its mRNA is localized in primordial follicles closest to the medullar region and labeling is more intense in the transitional and primary follicles. Thus the labeling pattern will appears as a gradient of signal, undetectable in the cortex and more intense toward the medullar region.

Figure 1 is the diagram showing a generalized overview of follicular development This diagram depicts the development of a dormant primordial follicle into a large mature follicle. Ultimately the mature follicle may be stimulated to ovulate and produce a fertilizable egg. This general process is the key for the production of eggs in many species.

Figure 2 is a schematic diagram representing the many potential steps and control points for follicular development. The ovary is depicted as a reservoir of dormant follicles and through the process of follicular development these dormant follicles will develop and mature to subsequently produce hundreds to thousands of eggs. Depicted is follicular development as a pipeline and the control of this process consisting of many valves or potential regulatory steps. In the later stages of follicular development, it is known that FSH is important for growth and development of mature antral follicles. Ultimately, LH stimulates the ovulations of a mature follicle and the production of the egg. However, the factors regulating early steps of follicular development have been essentially unknown for many years. The present invention was developed as an understanding how some of these early regulatory points are controlled in ovarian follicular development.

Figure 3 is a diagram showing the later stages of follicular development that occur in growing follicles when they respond to stimulation by FSH where they mature into antral follicles. These are readily observable by morphological changes in histological sections of ovaries. However, in the early stages of follicular development the morphological changes are not as obvious during the activation of dormant primordial follicles. Before questions about the regulation of the activation and development of these early stage follicles could be focused. Identity of genetic markers which could be detected and measure molecular changes in activated primordial follicles was necessary. These markers are genes that are turned on in the process of activation of dormant follicles that can be observed before any morphological changes take place. One family of genes that was known to be expressed in early stages of follicular development is the one for the zona pellucida. The zona pellucida is the glycoprotein matrix (ring around egg) that surrounds the mature oocyte. Note the lack of

protein matrices which surrounds the egg in the dormant follicle and the outer line in the growing follicle that indicates the presence of zona pellucida.

Table 1 lists all the genes for zona pellucida proteins that are present in four species. the rabbit, pig, mouse, and human. These have all been cloned and published. The zona pellucida gene that was chosen to investigate was the R55 gene in rabbit. The homologue this gene in the pig is ZP3α and there are similar genes present in the mouse and a human known as ZP1. First studies were designed and carried out in the rabbit so those studies are described first; R55was studied to determine if this gene fits criteria as a early to marker or indicator for activation of dormant primordial follicles.

TABLE 1

ZP Nomenclature

Rabbit:	R45, R55, and R75
Pig:	ZP3β, ZP3α, ZP2 and ZP1
Mouse:	ZP3, ZP2, and ZP1
Human:	ZP3, ZP2 and ZP1

Figure 4 shows the different stages of follicular development that are present in prepubertal rabbits. This is one of the reasons this species was selected for study. In prepubertal rabbits, ovarian development occurs after birth (humans are more like pigs in their temporal developmental profile). Because of this fact, ovaries from different ages of prepubertal rabbits were taken and with more mature follicles at each age. As shown in the fourteen day post-partum rabbit (which means fourteen days after birth) the ovary is essentially a bag of primordial follicles. At 28 days post-partum, a group of follicles have begun to develop and resulting in a population of primary follicles, which are morphologically different from the primordial follicles in that they contain a single layer of cuboidal granulosa cells surrounding the follicle. 42 days post-partum secondary follicles are present indicating yet another step of maturation in the early development of follicles. Moreover, by 56 days post-partum, tertiary follicles begin to appear which represent early stages of antral development in the ovarian follicles. The expression of the R55gene is linked to the activation in early development of ovarian follicles, *i.e.* its pattern of expression following this initial wave of follicular genesis.

Figure 5 graphically demonstrates the first wave of follicular development in prepubertal rabbits. A peak in the percentage of follicles initiating development (primary follicles) is seen around 6 weeks of age. These data are based on morphological characterization of histological sections and are presented in Table 2.

TABLE 2

Percentage of follicles present in rabbit ovaries of different ages.

	2wk	4wk	6wk	8wk
Primary	0	11	19	4
Secondary	0	0	6	11
Tertiary	0	0	0	3
Primordial	100	89	75	80

Figure 6 shows the northern blot analysis of R55 in immature rabbit ovaries. In this study. total RNA was isolated from different age rabbit ovaries, separated on agarose gels and transferred to biotrans nylon membranes. The membrane was then probed with a cDNA probe for R55 labeled with P^{35} and developed by autoradiography. As shown in panel A, R55 is undetectable at 14 days postpartum. A more intense band at 28 days postpartum and a very dark band at 42 days postpartum is presented. The R55 band is less intense at *56* days postpartum. This blot was then stripped and reprobed with a constitutive gene EF1α to show equal loading and transfer of the RNA. This experiment was repeated with 3 different groups of RNA. The intensity of the bands were determined by optical density and the data shown graphically.

Figure 7 is a graphic showing R55 expression in developing rabbit ovaries. The pattern of R55 expression correlates with the pattern from the first wave of folliculogenesis in the prepubertal rabbits as shown in Figure 5. This graph was obtained by determining the ratio of R55 to EF1α from each sample and then this number was graphed as a relative percentage of the maximum R55 expression found. Data supporting the graph of Figure 7 are presented in Table 3.

TABLE 3

R55 expression in rabbit ovaries from different ages.
Percent of maximum expression of R55 mRNA was determined on Northern blots.
Maximum expression occurs at 42 days postpartum (d.pp.)

d.pp.	14	28	42	56
% Max R55	3.1	36	100	45
SEM	1.8	9.8	0	7.3

Figure 8 is a composite showing the localization of R55 protein and messenger RNA in developing ovaries of prepubertal rabbits. Column 1 is the localization of R55 protein as determined by antibody staining with DAB conjugate. Columns 2 & 3 are in situ hybridizations for R55 messenger RNA. Column 4 is the in situ control using the sense strand as the riboprobe. The anti-sense strand was used in columns 2 and 3, all of which were labeled with S^{35}.

In the first row a single primordial follicle labeled for R55 protein and mRNA is shown. Based on hypothesis it is shown that this primordial follicle although it has not undergone any morphological changes has begun to develop since it is it expressing the R55 gene.

In the second row, it is shown that the primary follicles that appear by 28 days postpartum are expressing significant amounts of R55 protein and mRNA. Also important in this section shown by the arrow is a follicle, which we would term to be an intermediate follicle. It is somewhere in transition between the stages of primordial and primary and again as we would expect it is expressing a significant amount of R55. This is consistent with our prediction. In the third row is a section taken from a six week old rabbit (42 days postpartum). In this section, we show a secondary follicle that contains abundant amounts of zona pellucida protein beginning to form in a matrix around the oocyte in the first panel.

In the second and third panels, it is shown that the oocyte contains a large quantity of mRNA for R55. Not shown in this section are the many primary follicles that are present in 42 days postpartum that are also expressing abundant levels of R55. At 56 days postpartum in the fourth row, a developing follicle is shown and by this stage, a thick well developed matrix has formed around the oocyte that is a dark layer around the oocyte. In the second and third panels, it is shown that the amount of mRNA for R55 is dramatically decreased from what is seen in secondary follicles.

Returning to initial study steps of follicular development, a marker R55is initially expressed in activated follicles in the rabbit ovary. Figure 9 shows that it was determined that a second ZP gene in the rabbit R75 is a valid marker for activation for primordial follicles in its pattern of expression is similar to P55 so this gives two genes which can be measured in follicles to determine whether they are activated or not. Additionally, in rabbit two other genes were identified which are specific to granulosa cells and are expressed in these cells

during the transition from primordial to primary follicles. (Cx43 (connexin 43) and Inhibin-alpha). Now with these markers in hand questions about potential regulatory factors that might stimulate or inhibit activation dormant primordial follicles were considered.

Figure 10 shows the effects of growth factors EGF on R55 expression in immature rabbit ovaries. Ovaries were collected from 2 week old rabbits and the tissue mince into small pieces (-1mm^3). These ovarian explants were then cultured for 6 days in the presence or absence of EGF *(50* ng/ml). Total RNA was isolated and the relative amount of R55mRNA measured by Northern blot analysis. The result of 3 experiments were averaged and graphed. The level of P55 expression in the controls was defined as one. The level of expression of the epidermal growth factor treated samples was approximately 3 fold of that seen in the controls. This indicates that with treatment of epidermal growth factor there was a significant increase in the expression of R55 in these ovarian cultures. This effect is due to an increase in the number of follicles being activated. Data supporting the graph of Figure 10 are presented in Table 4.

TABLE 4

Results of 3 experiments showing stimulation of R55 mRNA expression by EGF. Values are the ratio of R55 to 28S optical densities from Northern blots.

	Control	EGF (50 ng/ml)
Exp. 1	1	2.87
Exp. 2	1	3.09
Exp. 3	1	3.28
Average	1	3.08
Std dev.	0	0.20518285
SEM	0	0.11846237

Figure 11 is directed to ovarian development in prepubertal pigs to determine if the inventive method would be applicable in other species. Figure 11 shows comparison of 10 week old pig ovaries to ovaries that are typical of a mature sow. In the rabbit, much (but not all) of the ovarian development occurs after birth. In the pig, ovarian development overlaps both the rabbit and human in developmental timetable. Development occurs over an extended time such that at 10 weeks the ovaries are very small and relatively immature. In pigs, antral follicles are normally not seen until 140 to 150 days (20 weeks) of age. This timetable may be accelerated by application of systemic EGF.

Figure 12 shows the follicle populations present in the immature pig ovaries at day 70 postpartum or ten weeks of age. In the two panels on the left, it is shown that a large population of primordial follicles are present in the cortex of ten week old pig ovaries. These represent the dormant or resting pool of primordial follicles, which will ultimately give rise to all the eggs that the gilt will produce throughout her life. In the two panels to the right, it is seen that in these same ovaries many secondary follicles are growing and developing. Based on morphology the 10 week pig ovary is very similar to the 42 day postpartum rabbit ovary.

Figure 13 diagrammatically depicts the follicular development that occurs during ovarian maturation in the pig. At birth, the ovary is primarily filled with dormant primordial follicles. Through the first 5 months of the gilts life the ovary matures and develops through the stimulation of endogenous growth factors and hormones such that at 5 months of age large population of antral follicles exists. These antral follicles are ready to be ovulated and will produce the eggs that are ovulated during the first heat.

Figure 14 shows the reproductive maturation timeline as relative to the situation found with commercial gilts. As depicted from birth through the first 5 months the ovary develops and matures. At about this time the gilt will undergo her first heat. At the first heat there is typically 10 to 12 eggs ovulated. After another 21 days, the gilt will enter her second heat at which time there will be slightly more eggs ovulated, on the order of 14 to 16 eggs. After another 21 days, the gilt will enter her third heat at which there will be 20 to 22 eggs ovulated and at this point, it is the recommended time for first breeding of a gilt. In this diagram, we can emphasize two obvious goals for the inventive method to accelerate ovarian maturation. One would be to increase the number of eggs ovulated in the first or second heat, such that commercial pork producers could then breed at these earlier times. Thus., saving time and feed costs while getting the most (larger litters) out of their immature gilts. Secondly, the inventive process could potentially shorten the period of time, the 5 month period, the gilt requires to reach the first heat. Both of these results combined would result in significant savings for the pork producer, with a concomitant increase in pigs per liter earlier in the reproductive cycle of the pig.

Figure 15 shows the inventive results established in the rabbit was applicable to the pig and it was determined to look at the effect of growth factors, specifically EGF, on ZP expression in immature pig ovaries. For this first experiment, ovaries were collected form pigs at three to five weeks of age at which the pig ovaries are very immature. The ovaries

were then minced into small pieces and grown in ovarian explant cultures with or without EGF (similar to what we have done with the rabbit ovaries). After 6 days of culture the tissue was collected and in this case proteins from these samples were isolated and solubilized. The amount of total zona pellucida protein in these samples was determined using an antibody we developed in guinea pigs against total zona pellucida proteins. The amount of porcine zona pellucida proteins in these samples were determined by dot blot analysis and quantified by optical density. The relative amount of zona pellucida protein is graphed for each sample (control and EGF treated 50 ng/ml). This was done for 3 replicates. These studies resulted in approximately a two fold increase in the amount of zona pellucida protein in the EGF treated ovarian explants. These results were consistent with the stimulation of P55 expression which shown in the rabbit ovarian explants. Data supporting the graph of Figure 15 is presented in Table 5.

TABLE 5

Stimulation of pig ZP proteins with EGF. Optical density of ZP proteins from ovarian explant cultures as measured by Protein dot blot analysis.		
Group ZP	OE EGF 50	OE control
	6355	2493
	6086	3763
	7193	3160
Avg. ZP	6544.66667	3 138.66667
Std. Dev.	577.358063	635.26871
SEM	333.337833	366.77256

Figure 16 graphically presents the results of studying the effect of EGF on ZP expression in isolated primordial follicles. As in the previous experiment, ovaries were collected from immature pigs at 3 to 5 weeks of age. In this case the ovaries were minced and the follicles enzymatically separated from the connective tissues and isolated. The population of small primordial follicles were collected and grown in cell culture well inserts. These were cultured for six days in medium alone as control or in medium supplemented with 50 nanograms per ml of EGF. After 6 days of culture the follicles were collected and proteins isolated and solubilized. The amount of total zona pellucida protein in these samples was determined using an antibody developed in guinea pigs against total zona pellucida proteins. The amount of porcine zona pellucida proteins in these samples were determined by dot blot

analysis and quantified by optical density. The relative amount of zona pellucida protein is graphed in Figure 16 for each sample (control and EGF treated 50 ng/ml). This was done for 3 replicates. As predicted from the hypothesis there was a significant increase in the expression of zona pellucida proteins in the primordial follicles treated with EGF. This is consistent with the hypothesis that EGF can stimulate activation of dormant primordial follicles as indicated by the expression of zona pellucida genes. Data supporting Figure 16 is presented in Table 6.

TABLE 6

Stimulation of pig ZP proteins in isolated primordial follicles with EGF. Optical density of ZP proteins from cultured primordial follicles as measured by Protein dot blot analysis.		
Group ZP	Primordial & EGF 50 ng/ml	Primordial, control
	1815	230
	371	9.85
	1697	0
Avg. ZP	1294.33333	79.95
Std. dev	801.8038	130.040407
SEM	462.92164	75.0788641

Figure 17 summarizes overall basic working model for acceleration of follicular development. Growth factors, specifically EGF, applied in early follicular development results in the acceleration of this process. In other words EGF stimulates activation of dormant follicles and accelerates follicular development at early growth stages of the animal. Increased numbers of activated follicles yield an increased number of follicles present and potentially available for maturation under the stimulation of FSH into large mature antral follicles, which at the LH surge would be ovulated. The result is the production of increased numbers of ovulated eggs and a shorter period of follicular development. This process can be applied to prepubertal development in the gilt (young sow) or other vertebrates to initiate or accelerate ovarian maturation and result in two positive effects. One being the increase in the number of eggs in early heats for the gilts and other species and two, possibly a shorter period of time to reach the first heat in these animals such that pork producers may be able to breed earlier and get more pigs per litter earlier in these animals.

Figure 18 is the DNA and amino acid sequences of encoding rec-pEGF protein. Rec-pEGF was expressed in the QiaExpress plasmid vector pQE30 . The DNA sequence

encoding pEGF begins at the adenine residue 36, with the AAT codon, encoding N^{13}, and ends at the cytosine residue 193 and the codon encoding Tyr^{65}. The DNA and amino acid sequence contains the 6X histadine tag at the amino-terminal end, and 19 amino acids from the bacterial plasmid vector at the COOH-terminal end. The 53 amino acids of the rec-pEGF is 100% identical to the published sequence for rec-pEGF published by Pascall, J.C., et al., *J. Mol. Endocrinol.* (1991) 6:63-70, which is hereby incorporated by reference as teaching a method for obtaining rec-pEGF using yeast and bacteriophage vectors.

Figure 19 is the results of gel electrophoresis on 15% polyacrylamide SDS gels, of expressed rec-pEGF protein purified on a Ni-Agarose column, showing a representative sample of some of the gels showing two purified samples of rec-pEGF. Lane 1 is a gel showing flow through of unbound bacterial proteins from a first sample run. Lane 2 is a gel showing the results after passing a wash buffer through the column. Lane 3 shows a relatively pure sample of rec-pEGF at a relative molecular weight of about 10 kDa, corresponding to the predicted molecular weight from the amino acid sequence of Figure 18. Lane 4 is a flow through sample of unbound bacterial proteins from a second sample run. Lane 5 is a gel showing the results after a wash buffer is passed through the column. Lane 6 shows a relatively pure sample of rec-pEGF at a relative molecular weight of about 10 kDa, corresponding to the predicted molecular weight from the amino acid sequence of Figure 18. Finally, lane 8 is the molecular weight markers.

Figure 20 is a Western blot assay performed to verify the identify of the rec-pEGF obtained by gel electrophoresis. The protein was transferred to an Immobilon membrane and analyzed with commercially available antibodies to mouse EGF. Panel A illustrates a coomassie stained gel of rec-pEGF and mouse EGF on a 1D-PAGE gel, and panel B illustrates the rec-pEGF and mouse EGF proteins which are recognized by rabbit anti-mouse EGF (Upstate Biotechnology, Incorporated) and detected which chemiluminescence. In both panels A and B, lane 1 is a molecular weight marker, lane 2 is 5.5 μg of rec-pEGF, and lane 3 is 0.25 μg mouse EGF.

In Figure 21, the bioactivity of EGF was tested in a 3T3 fibroblast proliferation assay. EGF was added to quiescent Swiss 3T3 fibroblast cells and stimulation of fibroblast proliferation was measured by $[H^3]$-thymidine incorporation as an indicator of DNA synthesis. Stimulation of fibroblast proliferation by rec-pEGF was comparable to that of purified mouse EGF.

Figure 22 is a graph comparing the average size of the ten largest follicles observed in histological sections of ovarian obtained from necropsy samples of the pigs in Group 1, above, comparing follicle size to that of the control group and the group administered nothing. Follicular diameter was measured using an ocular micrometer and only sections through the center of a follicle, having the germinal vesicle present, were used for the evaluation. It will be appreciated that the follicle size in the pigs given rec-pEGF were over 100% greater in diameter than that of the control group and over 70% greater than that of the group given nothing. Similarly, Table 7, below, offers a comparison of the organ weights of control and EGF-treated pigs in Group 2, and demonstrate the lack of effect of rec-pEGF on organ weights, body weights and general pathology.

TABLE 7

Measure	Control N=3	EGF N=3	Treatment P Value
Day 14 Weight (kg)	4.9 ± 0.24	4.6 ± 0.24	0.46
Day 70 Weight (kg)	24.5 ± 3.40	22.7 ±3.40	0.72
Avg. Daily Gain (kg/d)	0.35 ± 0.06	0.32 ± 0.06	0.76
Heart (gm)	124.4 ± 11.3	114.4 ± 11.3	0.56
Heart (% Body Weight)	0.52 ± 0.03	0.51 ± 0.03	0.91
Spleen (gm)	129.6 ± 38.1	112.0 ± 38.1	0.76
Spleen (% Body Weight)	0.53 ± 0.13	0.48 ± 0.13	0.82
Liver (gm)	703.9 ± 71.3	622.6 ± 71.3	0.50
Liver (% Body Weight)	2.91 ± 0.16	2.77 ± 0.16	0.57
Uterus (gm)	8.06 ± 0.82	8.15 ± 0.82	0.94
Uterus (% Body Weight)	0.034 ± 0.006	0.037 ± 0.006	0.77
Ovary (gm)	0.18 ± 0.085	0.36 ± 0.86	0.21
Ovary (% Body Weight)	0.0008 ± 0.0003	0.0018 ± 0.0003	0.23
Kidney (gm)	151.8 ± 18.1	139.8 ±18.12	0.81
Kidney (% Body Weight)	0.62 ± 0.07	0.64 ± 0.74	0.86

Finally, Figure 23 is a graph that confirms that *in vivo* administration of rec-pEGF to the Group 1 pigs, above, starting at 30 d.pp. and administered for fourteen consecutive days at a 540 μg/day dose by placement of subcutaneous osmotic pumps in each pig. Ovaries from a sub-set of Group 1 pigs were examined at 70 d.pp. when the first cohort of growing follicles is normally expected to be present. The data demonstrates clearly that the number of ovarian follicles were significantly increased by application of rec-pEGF over the control group and the group administered nothing. Data was obtained by counting the number of hematoxylin/eosin stained follicles in a counting frame (960 x 1400 μm), and only sections through the center of a follicle with the germinal vesicle present were used for the evaluation. Growing follicles were identified based upon the presence of at least one cuboidal layer of granulosa cells surrounding the oocyte.

The data clearly supports the proposition that administration of exogenous EGF accelerates ovarian development, increases ovulation rate at an age earlier than in untreated mammals, and increases the rate of ovarian maturation in prepubertal vertebrates. Additionally RNA samples were transferred to nylon membranes and will be evaluated for relative amounts of Z3α mRNA and will conform an increased level of follicular analysis by Northern blot analysis.

Although the invention has been described with respect to specific embodiments, it should be appreciated that other embodiments employing the concept of the present invention are possible without departing from the scope of the invention. The invention, for example, is not intended to be limited to the specific mammals discussed and exemplified and disclosed in these embodiments; rather the invention is defined by the claims in equivalence thereof.

What is claimed is:

1. A method for regulating vertebrate ovarian maturation and function, comprising the step of providing an amount of Epidermal Growth Factor to prepubertal ovaries of female vertebrate species sufficient to stimulate primordial ovarian follicular development and activate dormant ovarian follicles.

2. The method according to Claim 1, wherein the vertebrate species is swine.

3. A method for accelerating the onset of puberty in a prepubertal vertebrates, comprising the step of administering an amount of Epidermal Growth Factor sufficient to stimulate primordial ovarian follicular development and activate dormant ovarian follicles.

4. The method according to Claim 3 wherein the vertebrate are mammals.

5. The method according to Claim 4 wherein the mammals are selected from the group consisting of bovine, equine, porcine, canine, feline, human, farm animals and zoo animals.

6. The method according to Claim 1, wherein the vertebrate is swine and the Epidermal Growth Factor is administered to the swine less than or equal to 50 days post partum to activate ovarian and follicular development.

7. The method according to Claim 1, wherein the Epidermal Growth Factor activation of dormant follicles is monitored by gene markers which are initially transcribed in the activated primordial follicles and increase through stages of follicular development.

8. The method according to Claim 7, wherein the zona pellucida genes R55 and R75 are markers, which are initially transcribed in, activated primordial follicles.

9. The method according to Claim 1, wherein activation of dormant follicles and expression of the zona pellucida gene are increased by at least 200% by treatment of prepubertal mammals with Epidermal Growth Factor.

10. The method according to Claim 1, wherein a zona pellucida gene is a marker for determining whether Epidermal Growth Factor stimulates activation of primordial follicles, thereby resulting in expression of genes by oocytes in early stages of follicular development.

11. The method according to Claim 10, wherein the zona pellucida gene is ZP3-alpha and the expressed gene is a ZP gene.

12. A method for promoting ovulation in vertebrate species in species of different ages, comprising the step of providing an amount of Epidermal Growth Factor sufficient to initiate ovarian folliculogenesis.

13. The method according to Claim 12, wherein the species is the human female.

14. A method for effecting or regulating vertebrate ovarian maturation, comprising the step of administering a therapeutically effective amount of Epidermal Growth Factor to prepubertal vertebrates at different ages before primordial ovarian follicular maturation.

15. The method according to Claim 14, wherein the vertebrates are selected from the group of mammals comprising bovine, equine, porcine, canine, feline, human, farm animals and zoo animals.

16. The method according to Claim 14, wherein the vertebrate species is swine and the Epidermal Growth Factor is administered to the swine before fifty days post partum.

17. A method for promoting ovulation in vertebrate species, comprising the step of providing a therapeutically effective amount of Epidermal Growth Factor.

Follicular Development

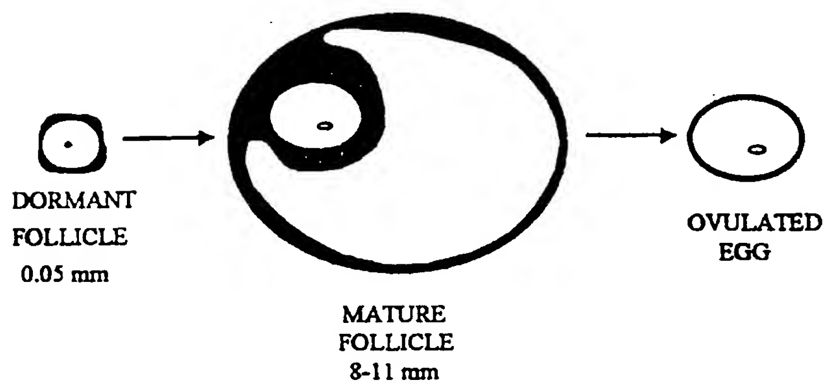


Fig. 1

How To Study Initial Steps of Follicular Development

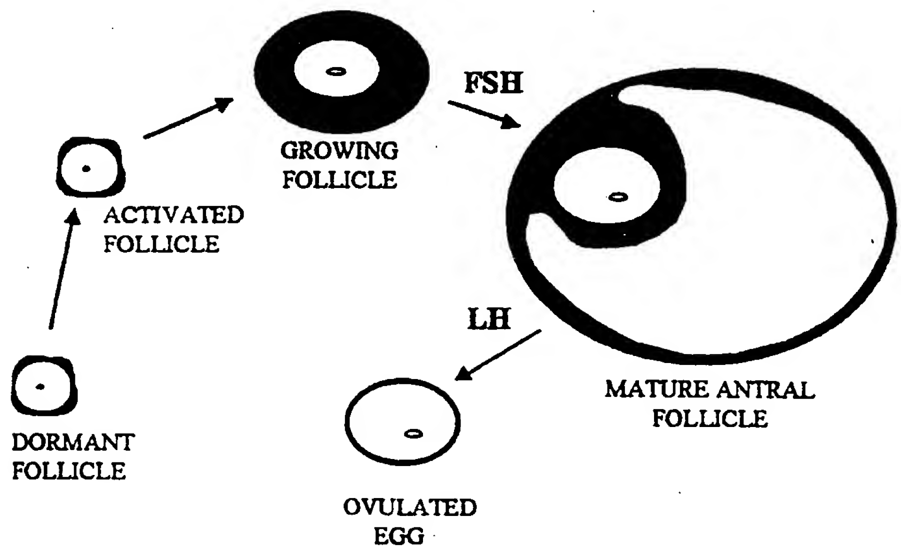


Fig. 3

Regulation of Follicular Development

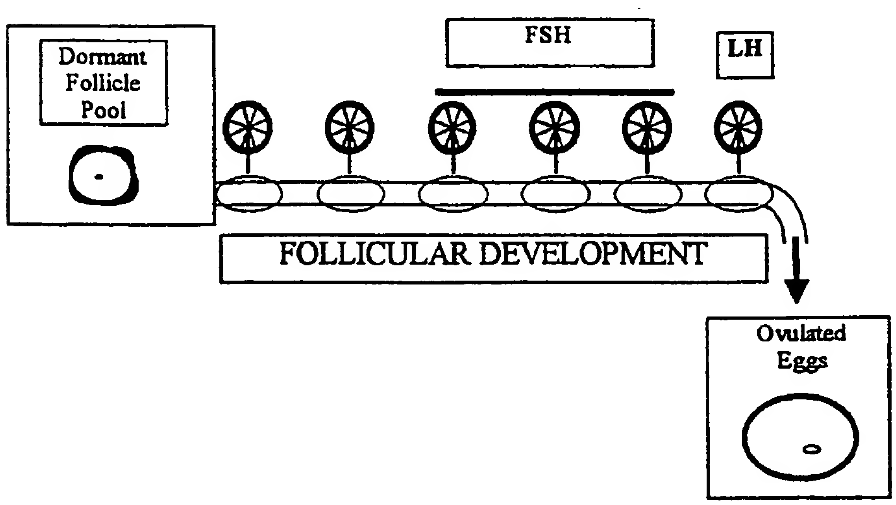


Fig. 2

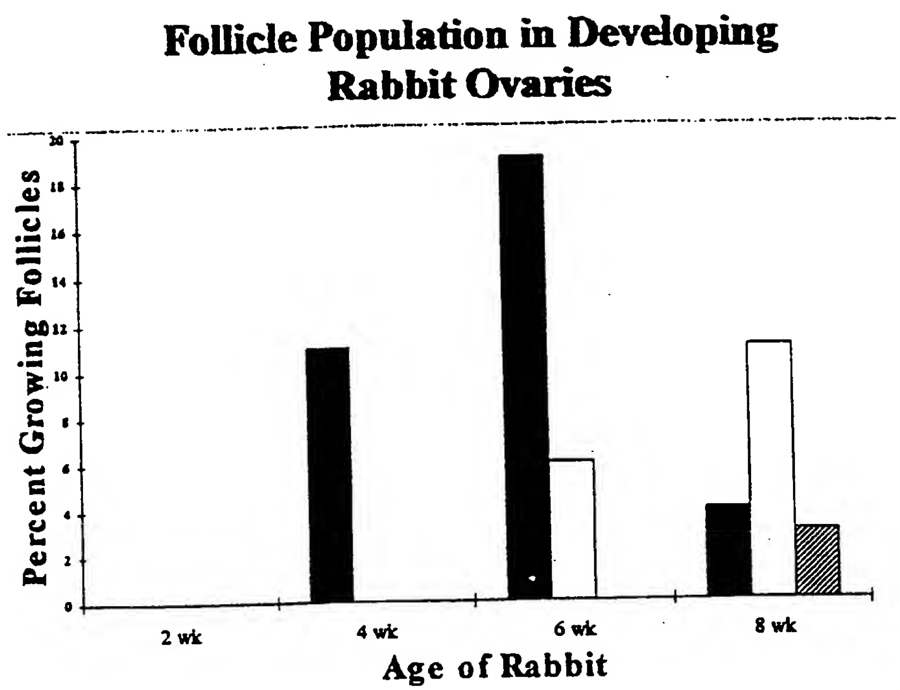

Follicle Population in Developing Rabbit Ovaries
Percent Growing Follicles
0
2
4
6
8
10
12
14
16
18
20
2 wk
4 wk
6 wk
8 wk
Age of Rabbit

Fig. 5

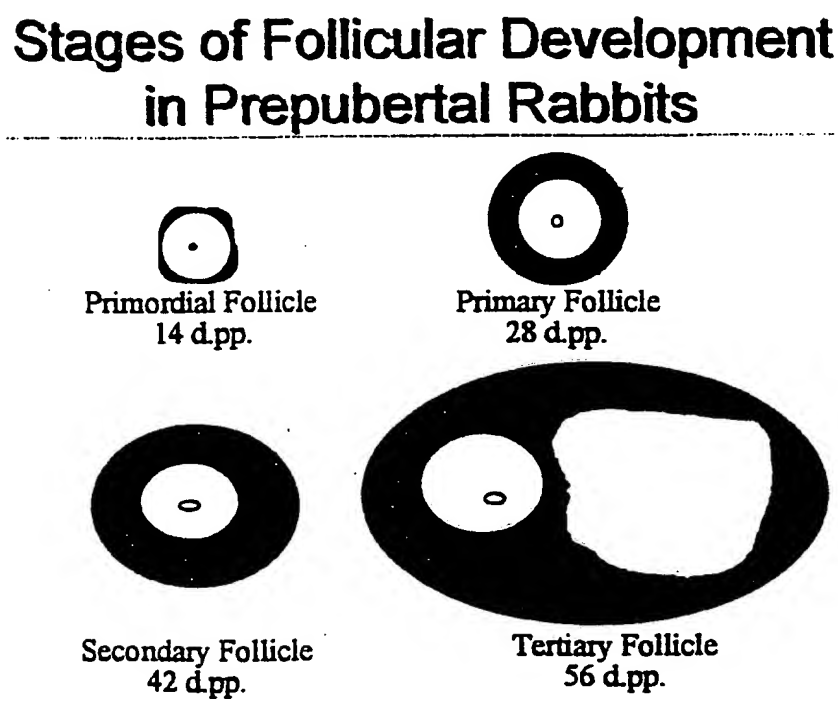

Stages of Follicular Development in Prepubertal Rabbits
Primordial Follicle
14 d.pp.
Primary Follicle
28 d.pp.
Secondary Follicle
42 d.pp.
Tertiary Follicle
56 d.pp.

FIG. 4

R55 Expression in Developing Rabbit Ovaries

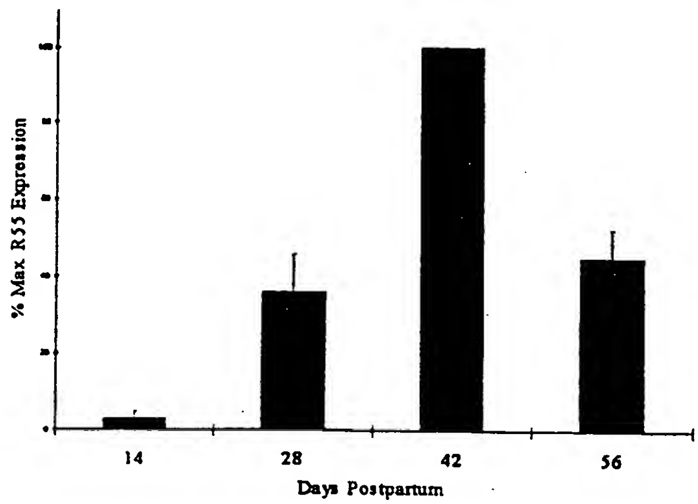


Fig. 7

Northern Blot Analysis of R55 in Immature Rabbit Ovaries

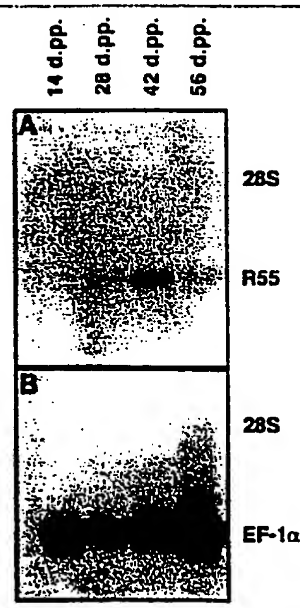


Fig. 6

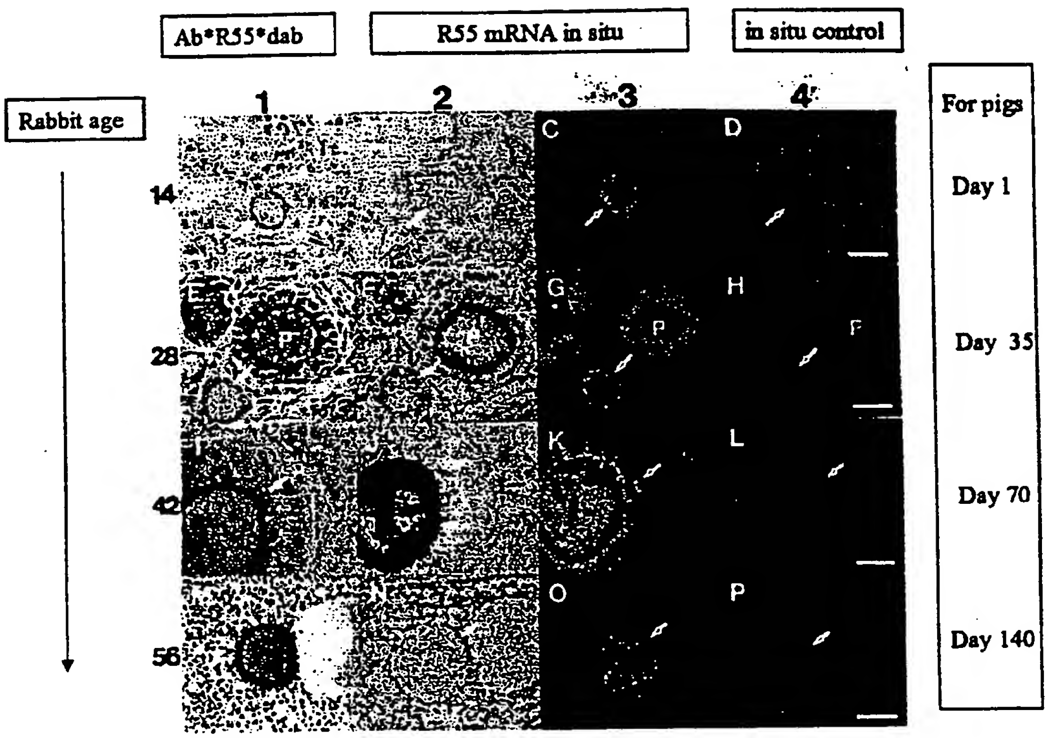

Ab*R55*dab
R55 mRNA in situ
in situ control
Rabbit age
1
2
3
4
For pigs
14
28
42
56
C
D
G
H
K
L
O
P
Day 1
Day 35
Day 70
Day 140

Fig. 8

How to Study Initial Steps of Follicular Development

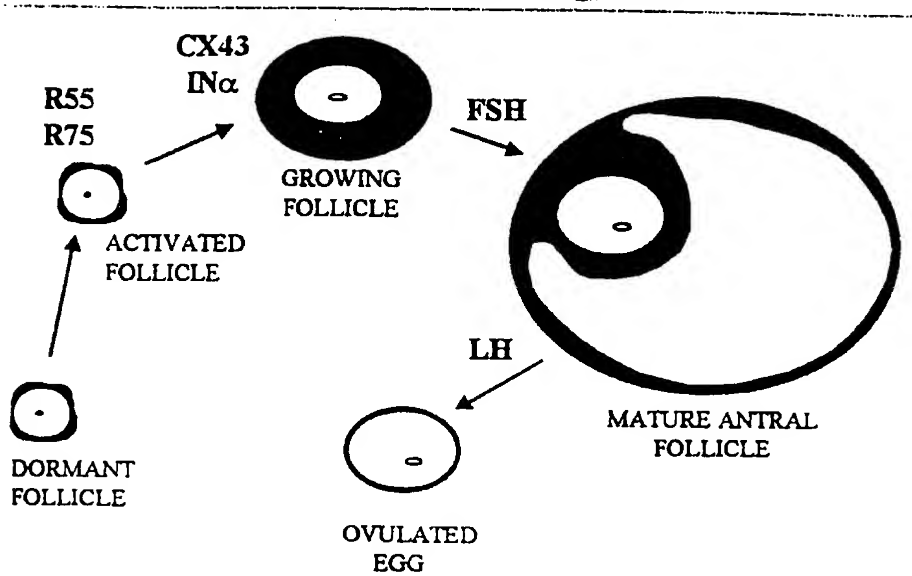


Fig. 9

Ovarian Maturation

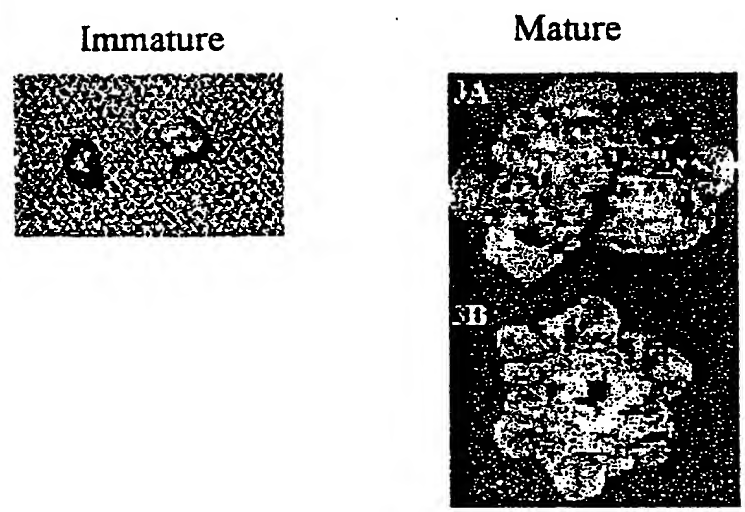


Fig. 11

Effect of Growth Factors on R55 Expression in Immature Rabbit Ovaries

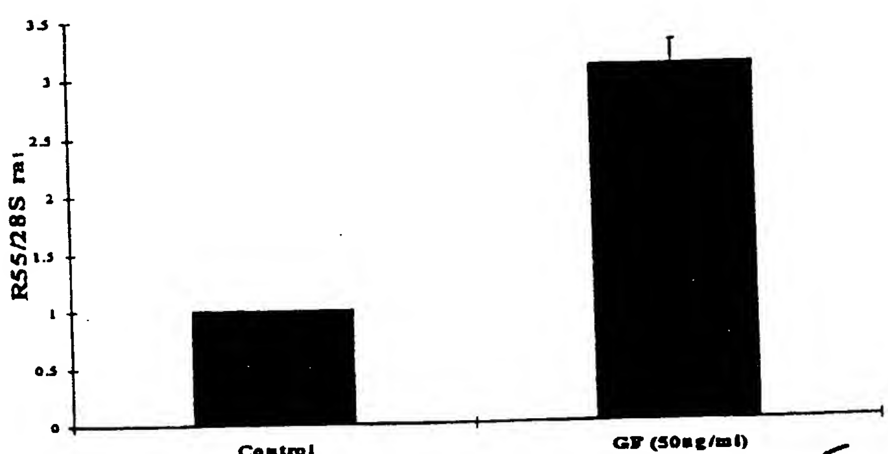


Fig. 10

Follicular Development During Ovarian Maturation

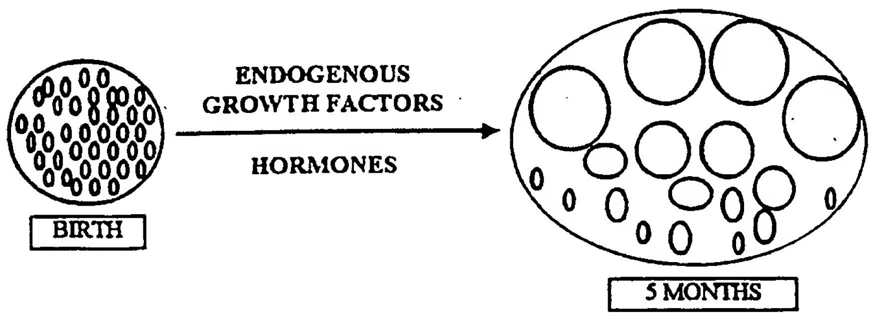


Fig. 13

Follicle Population in Immature Pig Ovaries Day 70 Postpartum

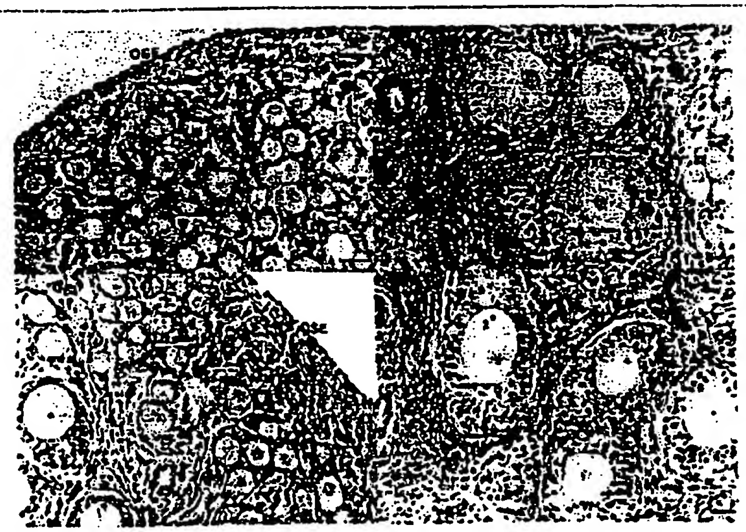


Fig. 1[illegible]

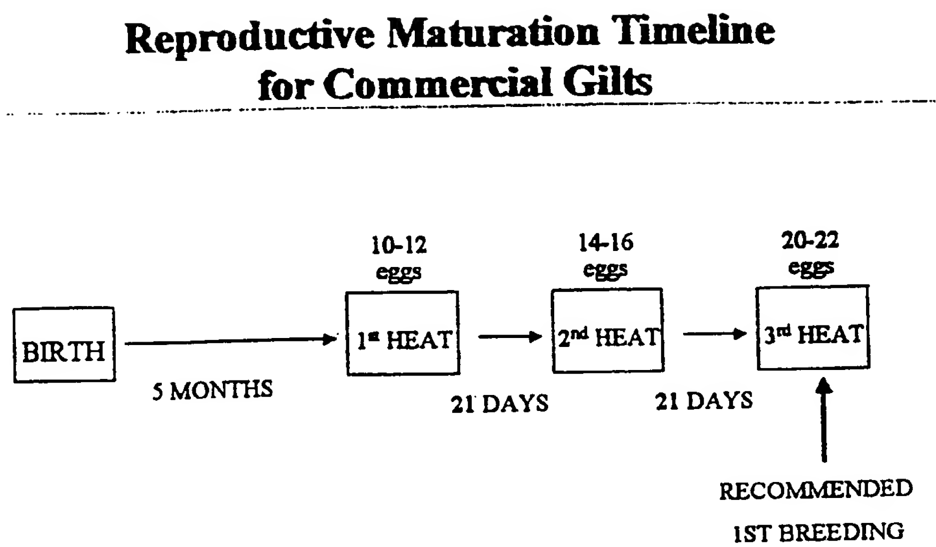

Reproductive Maturation Timeline
for Commercial Gilts
10-12
eggs
14-16
eggs
20-22
eggs
BIRTH
1st HEAT
2nd HEAT
3rd HEAT
5 MONTHS
21 DAYS
21 DAYS
RECOMMENDED
1ST BREEDING

Fig. 14

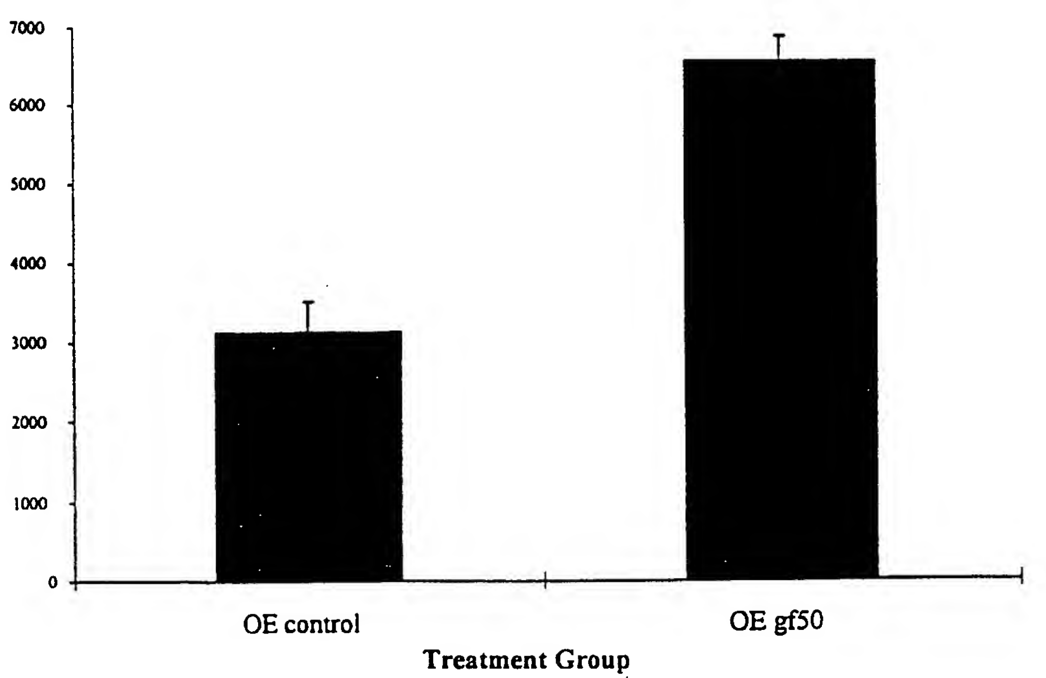

7000
6000
5000
4000
3000
2000
1000
0
OE control
OE gf50
Treatment Group

Fig. 15

Effect of Growth Factors on Follicular Development

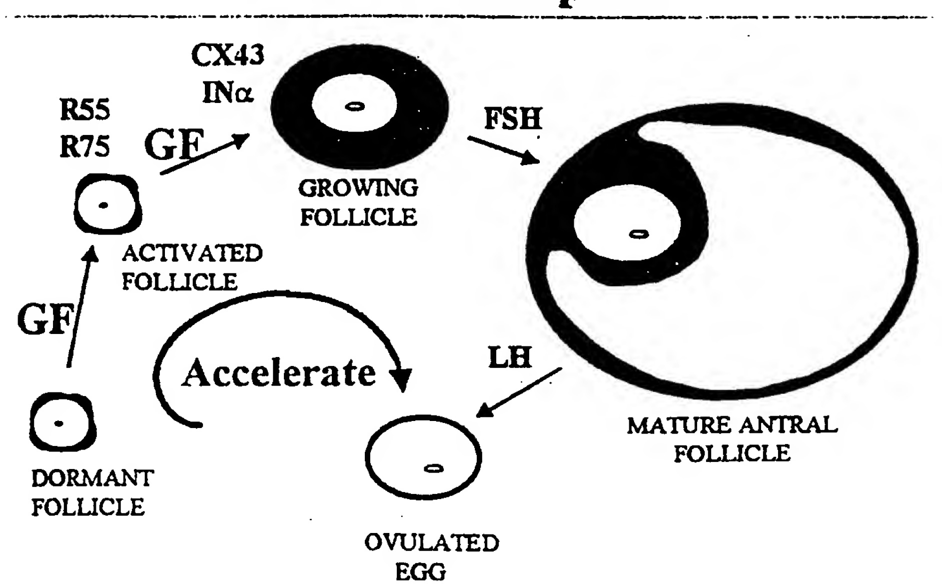


Fig. 17

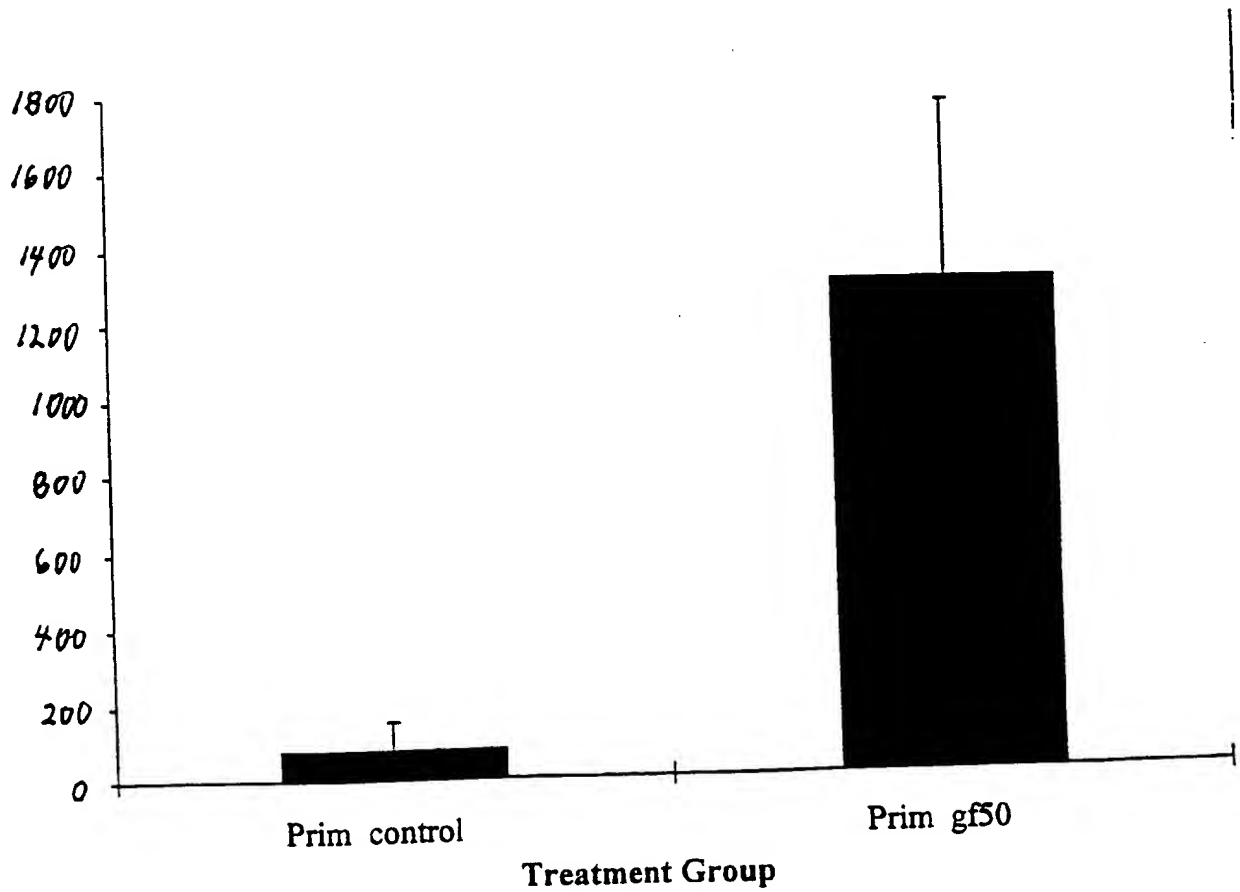

1800
1600
1400
1200
1000
800
600
400
200
0
Prim control
Prim gf50
Treatment Group

Fig. 16

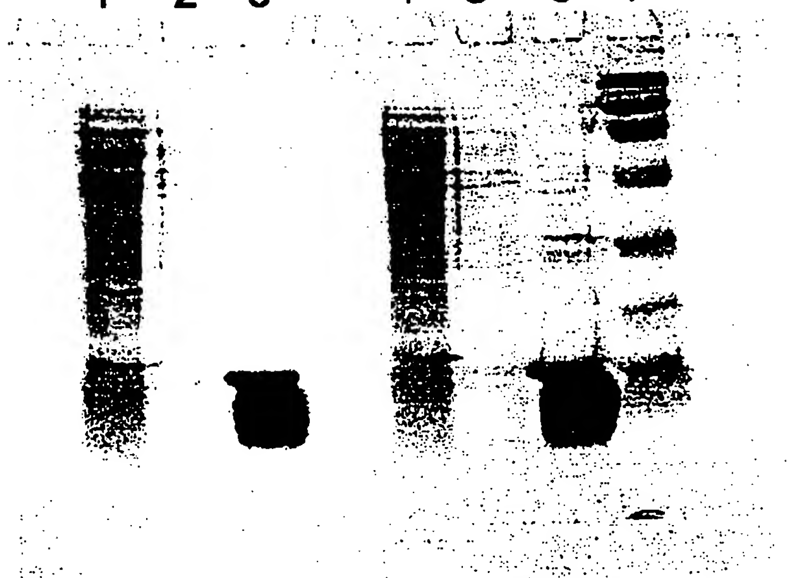
DNA and Amino Acid sequence of the recombinant pig EGF

July 23, 1998 13:20 ..

```
         ATGAGAGGATCGCATCACCATCACCATCACGGATCCAATAGTTACTCTGAATGCCCGCCG
      1  ---------+---------+---------+---------+---------+---------+ 60
         TACTCTCCTAGCGTAGTGGTAGTGGTAGTGCCTAGGTTATCAATGAGACTTACGGGCGGC

a        M  R  G  S  H  H  H  H  H  H  G  S  N  S  Y  S  E  C  P  P  -

         TCCCACGACGGGTACTGCCTCCACCGTGGTGTGTGTATGTATATTGAAGCCGTCGACAGC
     61  ---------+---------+---------+---------+---------+---------+ 120
         AGGGTGCTGCCCATGACGGAGGTGCCACCACACACATACATATAACTTCGGCAGCTGTCG

a        S  H  D  G  Y  C  L  H  G  G  V  C  M  Y  I  E  A  V  D  S  -

         TATGCCTGCAACTGTGTTTTTGGCTACGTTGGCGAGCGATGTCAGCACAGAGACTTGAAA
    121  ---------+---------+---------+---------+---------+---------+ 180
         ATACGGACGTTGACACAAAAACCGATGCAACCGCTCGCTACAGTCGTGTCTCTGAACTTT

a        Y  A  C  N  C  V  F  G  Y  V  G  E  R  C  Q  H  R  D  L  K  -

         TGGTGGGAGCTGCGCAAGCCGAATTCGAGCTCGTACCCGGGGTCCTCTAGAGTTGACCTG
    181  ---------+---------+---------+---------+---------+---------+ 240
         ACCACCCTCGACGCGTTCGGCTTAAGCTCGAGCATGGGCCCCAGGAGATCTCAACTGGAC

a        W  W  E  L  R  K  P  N  S  S  S  Y  P  G  S  S  R  V  D  L  -

         CAGCCAAGCCGATAG
    241  ---------+----- 255
         GTCGGTTCGGCTATC

a        Q  P  S  R  *  -
```

Figure 1. This is the DNA and amino acid sequences of the rec-pEGF protein being expressed in the bacterial system. It contains the 6X histadine tag at the NH4 terminal, the 53 amino acids of pig EGF (100% identical to published sequence for pig), and 19 amino acids from the bacterial plasmid vector.

FIG 18



Lane 1: LP6A-SP1 Flow Through (unbound bacterial proteins).
Lane 2: LP6A-SP1 Buffer Wash of column.
Lane 3: LP6A-SP1 Eluted rec-pEGF from column.
Lane 4: LP6B-SP1 Flow Through (unbound bacterial proteins).
Lane 5: LP6B-SP1 Buffer Wash of column.
Lane 6: LP6B-SP1 Eluted rec-pEGF from column.
Lane 7: Rainbow Molecular Weight Markers

Figure 2. The expressed rec-pEGF protein was purified on Ni-Agarose columns and analyzed on 15% polyacrylamide SDS gels. This picture is a representative sample of some of these gels showing two purified samples of rec-pEGF. Lanes 3 and 6 demonstrate a relatively pure sample of rec-pEGF at a relative molecular weight of ~10 kilodaltons (predicted molecular weight from amino acid sequence).

FIG. 19

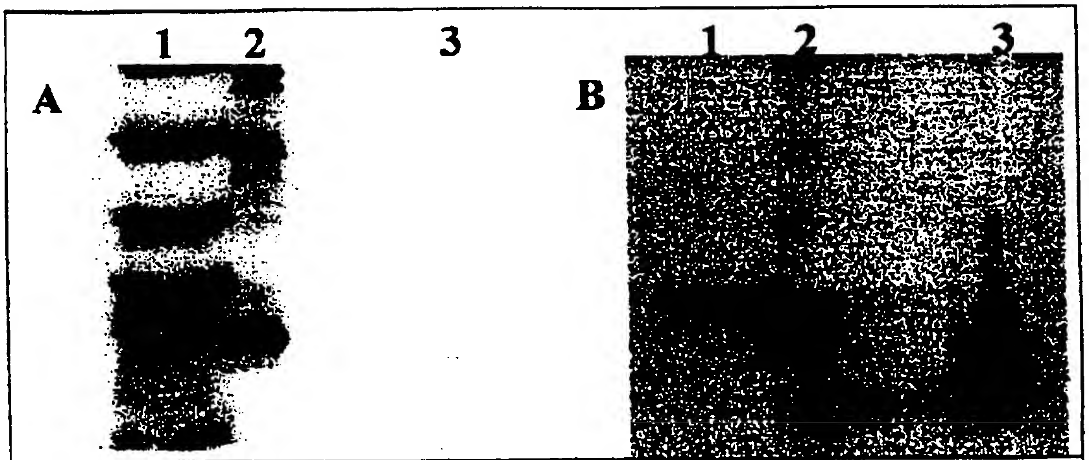


Figure 3. Western blot analysis of rec-pEGF. **A** is a coommassie stained 1D-PAGE gel and **B** is a Western blot probed with mouse EGF antibodies and detected with chemiluminescence. Lane 1: molecular weight marker; lane 2: 5.5 μg of rec-pEGF; lane 3: 0.25 μg mouse EGF.

FIG 2D

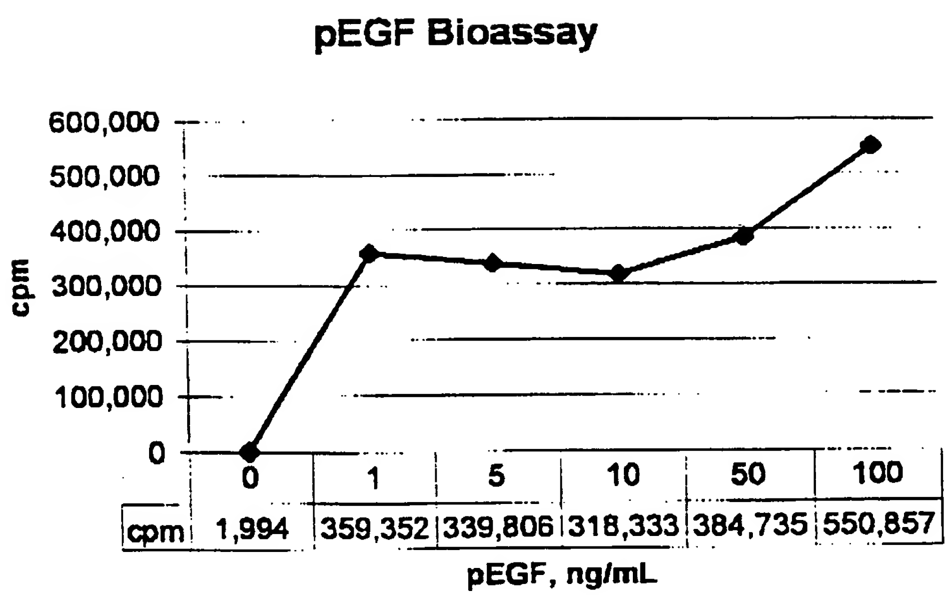


Figure 4. Bioassay for recombinant pig EGF (rec-pEGF). Note that increasing concentrations of pEGF cause an increase in uptake of tritiated tymidine.

FIG 21

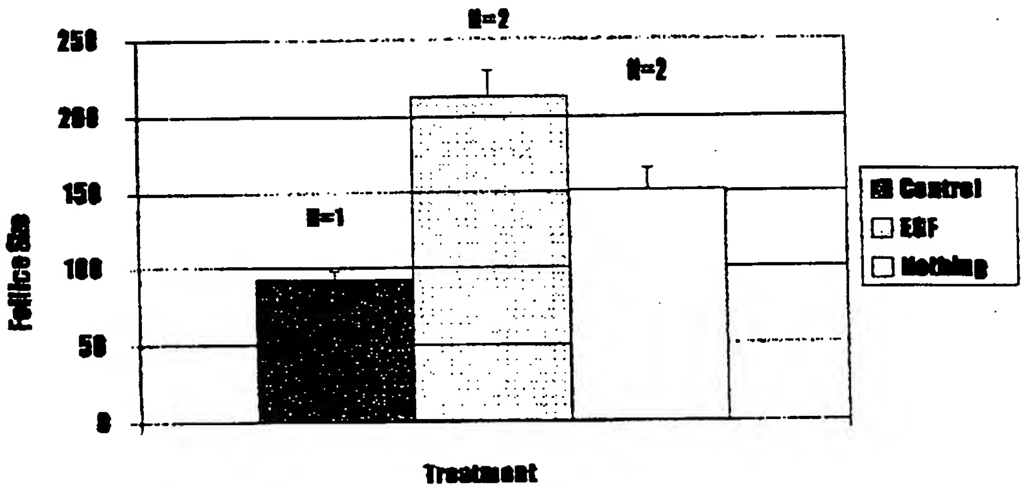


Figure 5. Graph of the average size of the ten largest follicles observed in H/E stained sections of ovaries from pigs in the pilot studies. Diameters of follicles were measured with an ocularmicrometer and only sections through the center of a follicle (germinal vesicle present) were used for these evaluations.

FIG 22

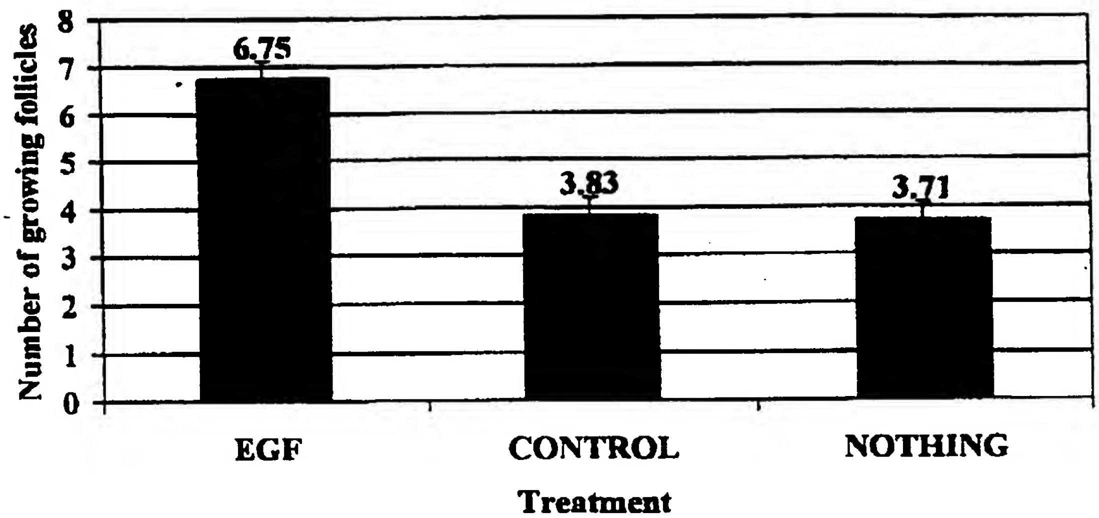


Figure 6. Numbers of follicles were increased by application of EGF. Ovaries were examined at 70 days of age when the first cohort of growing follicles are expected to be present. Graph of the average number of growing follicles in a defined area observed in H/E stained sections of ovaries from pigs in the pilot studies. Number of growing follicles were counted in a frame (960 x 1400 μm) and only sections through the center of a follicle (germinal vesicle present) were used for these evaluations. Growing follicles were identified based on the presence of at least one cuboidal layer of granulosa cells surrounding the oocyte.

FIG 23